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ERRATA

Vol. 6, page 157, table heading, "TABLE III (*Continued*)" should read "TABLE VIII (*Continued*)"

Vol. 7, page 57, Figure 1, line 2, "appled" should read "apple"

Vol. 11, page 323, line 3 from bottom, "**12**" should read "**13**"

Vol. 12, page 466, line 24, "emulsion" should read "emulsin"

Vol. 12, page 468, TABLE I, columns 5, 6, 7, and 8, "emulsion" should read "emulsin"

Vol. 12, page 479, Figure 7, extreme left of curve, point at "125" should be at "150" at the 10-day interval. For corrected curve see Figure 3 in Contrib. Boyce Thompson Inst. **13**: 505. 1945.

Vol. 13, page 367, line 15, "*niger*" should read "*majus*"

Vol. 13, page 401, TABLE IV, column 6, "$0.313\ V-0.025\ V^2$" should read "$0.313\ V-0.505\ V^2$"

Vol. 13, page 404, line 10 from bottom, "$-2\sqrt{\frac{(R'')^3}{A}}\ D=$ the slope of" should read "$-2\sqrt{\frac{(R'')^3}{A}}=$ the slope of"

Vol. 13, page 440, TABLE VI, footnote should read "The knockdown of the O.T.I. was almost complete in every case."

ADDENDA

Vol. 6, page 173, Figure 7, add "Temperatures used: upper left, 75° F.; upper right, 60° F.; lower left, 50° F.; lower right, 40° F."

Vol. 12, page 513, column 2, after line 12, add "Viruses: β-naphthoxyacetic acid-treated plants have temporary appearance similar to diseased, 1"

Vol. 13, page 355, add footnote[1] to title, "Abstract of this published *as* Carbon dioxide storage. XII. The ineffectiveness of carbon dioxide as an inhibitor of germination of seeds. *in* Amer. Jour. Bot. **24**: 734. 1937."

PROTECTING COTTON FIBER FROM PERIODATE OXIDATION[1]

RICHARD E. REEVES

The problem of protecting cotton fiber from certain types of oxidative degradation is one of considerable economic importance. It has long been recognized that some of the desirable properties of cottonseed fiber are profoundly affected by the action of small amounts of oxidizing agents (13, p. 143–144), but the chemical changes which accompany most types of fiber oxidation are so obscure that little is known regarding the position of attack or the nature of the oxidation products. There is, however, at least one oxidizing agent (periodic acid, H_5IO_6) suitable for use on cotton fiber the action of which has been found to be highly selective in that it preferentially oxidizes 1,2-glycol groupings. Experiments with the action of this reagent upon simple reference compounds have shown that substitution of methoxyl groups for one or both hydroxyl groups prevent the oxidation of the glycol (1, 2). The present work was undertaken to discover the extent of substitution for hydroxyl groups in cotton fiber which would be required to prevent or greatly diminish the oxidation of the fiber by periodic acid.

The oxidation of cotton fiber by small amounts of periodic acid appeared to offer certain advantages for initiating a study of possible methods for fiber protection. The oxidation will proceed under very mild conditions. The reaction is easily controlled, results are highly reproducible upon a given fiber preparation, and the chemical changes brought about in the fiber are probably less heterogeneous than those produced by many other oxidizing agents.

The drastic oxidation of cellulosic materials with periodic acid has been studied by Jackson and Hudson (11, 12) and by Grangaard, Gladding, and Purves (10). These workers have isolated some of the oxidized fragments and found them to be those anticipated from the recognized tendency of this reagent to cleave 1,2-glycols into fragments containing two carbonyl groupings. Davidson (6, p. 218; 7) and Brownsett and Davidson (3) have published certain observations upon the change in viscosity of cotton fiber upon mild oxidation with periodic acid. They have observed that the cuprammonium viscosity falls off markedly upon oxidation while nitrate viscosity (the viscosity in organic solvents of the nitrated cotton fiber) is only slightly changed by the oxidation. Recently Rutherford, Minor,

[1] A contribution from the former Cellulose Department of the Chemical Foundation, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York.

Martin, and Harris (15) have shown that if the oxidized fiber is treated with alkali before nitration the nitrate viscosity falls off in a manner corresponding to the loss of cuprammonium viscosity. These workers have concluded that periodate oxidation introduces alkali-labile linkages which are broken down by the highly alkaline cuprammonium solution.

In the present work only the action of small amounts of the oxidizing agent has been studied and the degradation has been followed by means of the viscosity of cuprammonium hydroxide dispersions of the oxidized fiber. Cuprammonium viscosity and its reciprocal, fluidity, are well recognized indices of cotton fiber quality (13, p. 85–94). The British Fabrics Research Committee (8, p. 6) lists six classes of cotton quality corresponding to different ranges of cuprammonium fluidity from 1 to 50 rhes (reciprocal poise) based on 0.5 per cent dispersions:

Fluidity, Rhes. ($100/\eta cp$)	Class
1 to 5	" . . . very mildly prepared . . . cottons.
5 to 10	. . . normal scoured and bleached cotton . . .
10 to 20	. . . textile cotton that has suffered a significant loss in its tensile strength . . .
20 to 30	. . . badly over-bleached textile cotton that suffered a serious loss of tensile strength.
30 to 40	. . . cottons which show incipient loss of fibrous structure . . .
40 to 50	. . . cottons highly degraded by chemical attack of the kind described as 'oxy and hydrocelluloses' in the older literature."

MATERIALS AND METHODS

The following experiments were made with mature fibers of *Gossypium hirsutum* L., Super Seven variety, Strain 4. Unless otherwise specified the fiber was extracted in a Soxhlet extractor for four hours each with alcohol-benzene (1:1) followed by water. Mercerized fiber was prepared from the extracted fiber by treatment at 20° C. with 25 per cent sodium hydroxide, followed by wash, sour, wash, and air drying.

Viscosity measurements were made in modified Clibbens and Geake type viscometers using 0.5 per cent dispersions of the fiber in cuprammonium containing approximately 15 g. copper and 240 g. ammonia per liter. A more detailed description of this procedure for determination of viscosity has been given by Compton (4).

Methylated cotton fiber was prepared by the use of diazomethane in ether at 0° C. (14). This procedure introduces the substituent with a minimum of fiber deterioration. X-ray diffraction patterns of the substituted fibers indicate that methyl groups so introduced are more extensively con-

fined to the non-crystalline portions of the cotton fiber than methyl groups introduced by other procedures.[2]

The desired dilutions of periodic acid were made from a 0.3 molar stock solution. Quantitative determinations of periodate employed the arsenite procedure described by Fleury and Lange (9). In certain instances this procedure was made more delicate by the use of 0.004 N in place of 0.1 N iodine solution.

Samples of air-dried cotton fiber were weighed to contain 162 mg. of anhydrous, unsubstituted fiber. (This amount was arbitrarily chosen because it corresponds to 1 millimol of glucose anhydride in the formula for pure cellulose.) This amount of fiber was placed in 5 cc. of aqueous solution containing a known amount of periodic acid. After standing at room temperature for an interval of time any remaining reagent was determined and the total consumed by the sample calculated. It was found that amounts of reagent up to and including 0.01 millimol were completely used by the unsubstituted samples within 24 hours. After oxidation the fibers were washed with water, dried in air, and reserved for viscosity measurements. Control experiments with equal or stronger concentrations of iodic and hydrochloric acid showed almost no change in viscosity, hence the observed effects can be attributed almost entirely to the oxidizing action of the reagent, and not to a hydrolytic action.

The viscometric measurements are expressed in centipoise units (ηcp) or in fluidity units called rhes (100/ηcp). The latter is a useful function in that increase in fluidity roughly parallels loss of textile quality. Since almost all of these dispersions exhibited the anomalous viscosity effect characteristic of cellulose dispersions (5), the values are "apparent" viscosities and are somewhat dependent upon the type of instrument in which the measurement is made.

THE OXIDATION OF UNSUBSTITUTED COTTON FIBER

It was observed that the behavior of a sample of cotton fiber upon oxidation with periodic acid is dependent upon the pre-treatment which the fiber has received. Raw cotton or benzene-alcohol extracted cotton fiber consumes a considerable amount of reagent before any fall in viscosity is noted. Water extracted fiber does not show this lag, presumably because a readily oxidizable substance is removed which had contributed little or nothing toward the viscosity. Mercerized fibers show extensive loss of viscosity upon oxidation with very small amounts of reagent. Oxidation at the proportion of one mole of reagent per 162,000 g. mercerized cotton fiber caused a lowering of the viscosity from 37.7 to 15.7 centipoise units. Rutherford and coworkers (15) have investigated the formation of aldehyde groups in cotton fiber upon oxidation with periodic acid and found

[2] Unpublished observations by Dr. Wayne A. Sisson.

that a small amount of reagent (0.002 millimol per 162 mg. fiber) disappeared from solution without producing aldehyde groups. They concluded that this amount of reagent was adsorbed by the fiber without reaction. In the present experiments as little as 0.0005 millimol of reagent per 162 mg. of water extracted or mercerized fiber produced marked changes in the viscosity, hence it appears that under these conditions the adsorption phenomenon does not remove the first 0.002 millimol of reagent without reaction. The change in cuprammonium viscosity of various fiber preparations upon oxidation with small amounts of periodic acid is shown in Table I and Figure 1.

TABLE I

CUPRAMMONIUM VISCOSITY OF RAW AND TREATED COTTON FIBER AFTER OXIDATION WITH PERIODIC ACID

Millimols H_5IO_6 consumed by 162 mg. anh. fiber in 24 hours	Raw cotton fiber ηcp	Pre-treatment of cotton fiber		
		Alcohol-benzene ext'd. ηcp	Water ext'd. ηcp	Mercerized ηcp
—	53.2	53.0	52.4	37.7
0.00025	—	—	—	35.2
0.0005	52.9	52.7	38.7	27.6
0.00075	—	—	—	20.3
0.0010	53.2	52.9	24.6	15.7
0.0015	—	—	16.6	—
0.002	53.3	40.4	12.4	7.9
0.003	39.7	31.2	8.5	6.5
0.004	33.4	—	—	4.3
0.005	23.5	17.1	5.3	3.7
0.007	11.9	—	—	—
0.01	7.6	—	—	2.3
0.02	3.5	—	—	—
0.05	1.9	—	—	—
5 cc. solution containing	ηcp after 24 hours			ηcp after 24 hours
0.1 mm. HCl	45.2			—
0.5 mm. HIO_3	—			33.2

COMPARISON OF THE RATE OF OXIDATION OF METHYLATED AND UNMETHYLATED COTTON FIBER

Periodic acid oxidation. Samples of alcohol-benzene and water extracted fiber and mercerized fiber were partially methylated with ethereal diazomethane and then subjected to oxidation by periodic acid with unmethylated fibers being exposed to the reagent for comparison under the same conditions of temperature and initial concentration. It was found that 9.8 per cent methoxyl in native fiber caused an appreciable decrease in the rate of oxidation while 19.1 per cent methoxyl in mercerized fiber showed roughly the same degree of protection. These two methylated preparations were superior to unmethylated samples in having lower rates

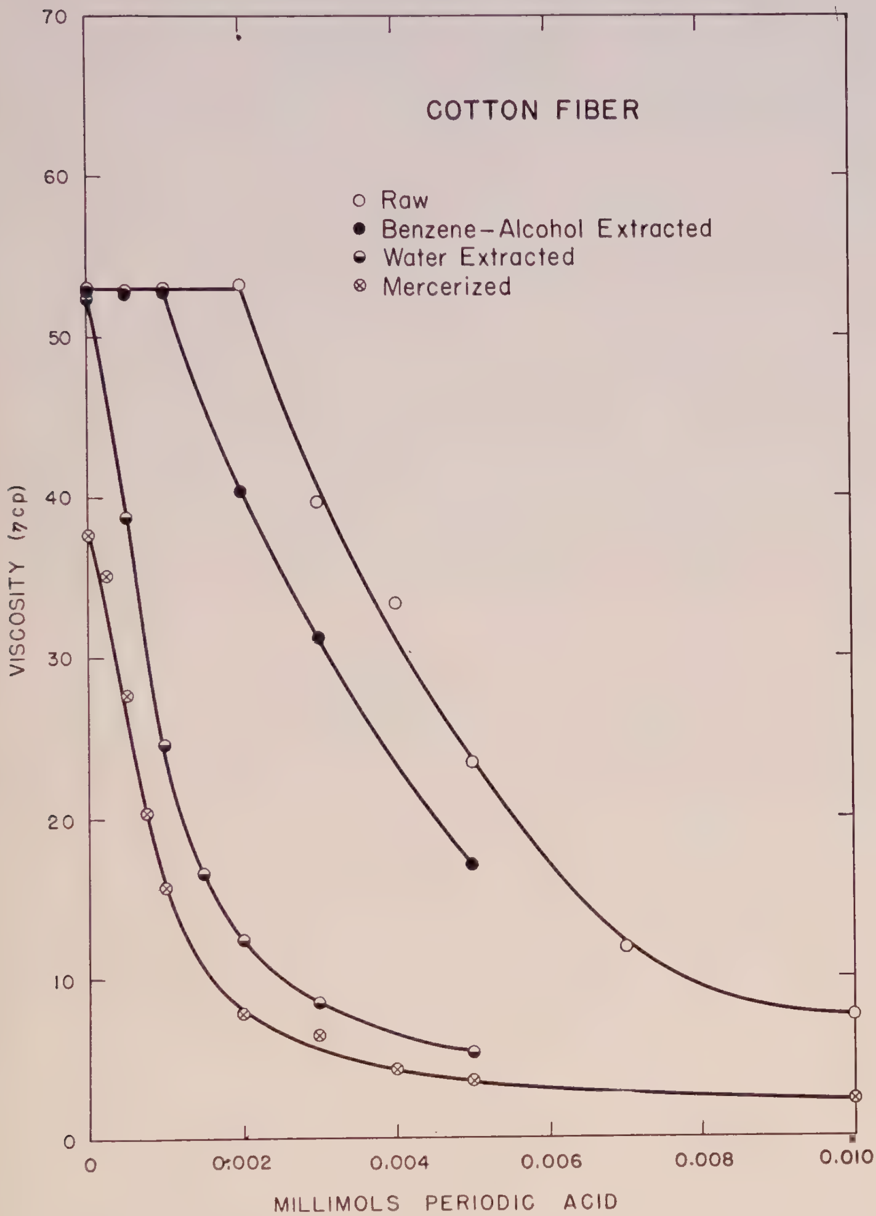

FIGURE 1. The cuprammonium viscosity of 162 mg. samples of cotton fiber after oxidation with periodic acid.

of reaction with the oxidizing agent and in showing less loss of viscosity during a specified interval of time. It required three hours for native cotton fiber to reach a fluidity of 20 rhes (the badly over-bleached textile cotton class) while the fiber containing 9.8 per cent methoxyl required 17 hours to reach this state. In the mercerized fibers the control reached 20 rhes in

TABLE II

EFFECT OF PARTIAL METHYLATION UPON THE OXIDATION OF NATIVE COTTON FIBER

Time of reaction at 25° C., hours	Oxidation with 5 cc. of 0.002 M H_5IO_6 per 162 mg. anhydrous, unsubstituted fiber					
	Unsubstituted fiber		3.33% Methoxyl		9.82% Methoxyl	
	Millimols H_5IO_6 consumed	ηcp	Millimols H_5IO_6 consumed	ηcp	Millimols H_5IO_6 consumed	ηcp
0	—	51.4	—	53.0	—	33.7
1.25	0.0040	7.7	0.0030	9.1	0.0018	21.3
5	0.0061	4.1	0.0052	4.3	0.0028	9.6
12	0.0084	3.6	0.0071	3.1	0.0032	5.9
24	0.0100	3.6	0.0088	2.8	0.0041	4.3

TABLE III

EFFECT OF PARTIAL METHYLATION UPON THE OXIDATION OF MERCERIZED COTTON FIBER

Time of reaction at 25° C., hours	Oxidation with 5 cc. of 0.002 M H_5IO_6 per 162 mg. anhydrous, unsubstituted fiber					
	Unsubstituted fiber		7.60% Methoxyl		19.1% Methoxyl	
	Millimols H_5IO_6 consumed	ηcp	Millimols H_5IO_6 consumed	ηcp	Millimols H_5IO_6 consumed	ηcp
0	—	43.7	—	22.1	—	36.1
1.25	0.0040	4.3	0.0028	5.0	0.0019	16.1
5	0.0076	2.7	0.0060	2.9	0.0031	7.2
12	0.0088	2.4	0.0086	—	0.0048	4.3
24	0.0100	2.4	0.0098	2.3	0.0073	3.3

approximately one and one-quarter hours while the sample containing 19.1 per cent methoxyl required nine hours. No appreciable protection was afforded by native fiber containing 3.3 per cent methoxyl or by mercerized fiber containing 7.7 per cent methoxyl. The experimental results are shown in Tables II and III, respectively. Figure 2 A and B shows a comparison between the fluidity changes of the more highly methylated native and mercerized fibers and their respective controls. The values 9.8 and 19.1 per cent methoxyl correspond roughly to the methylation of one-sixth and one-third of the hydroxyl groups of cotton fiber.

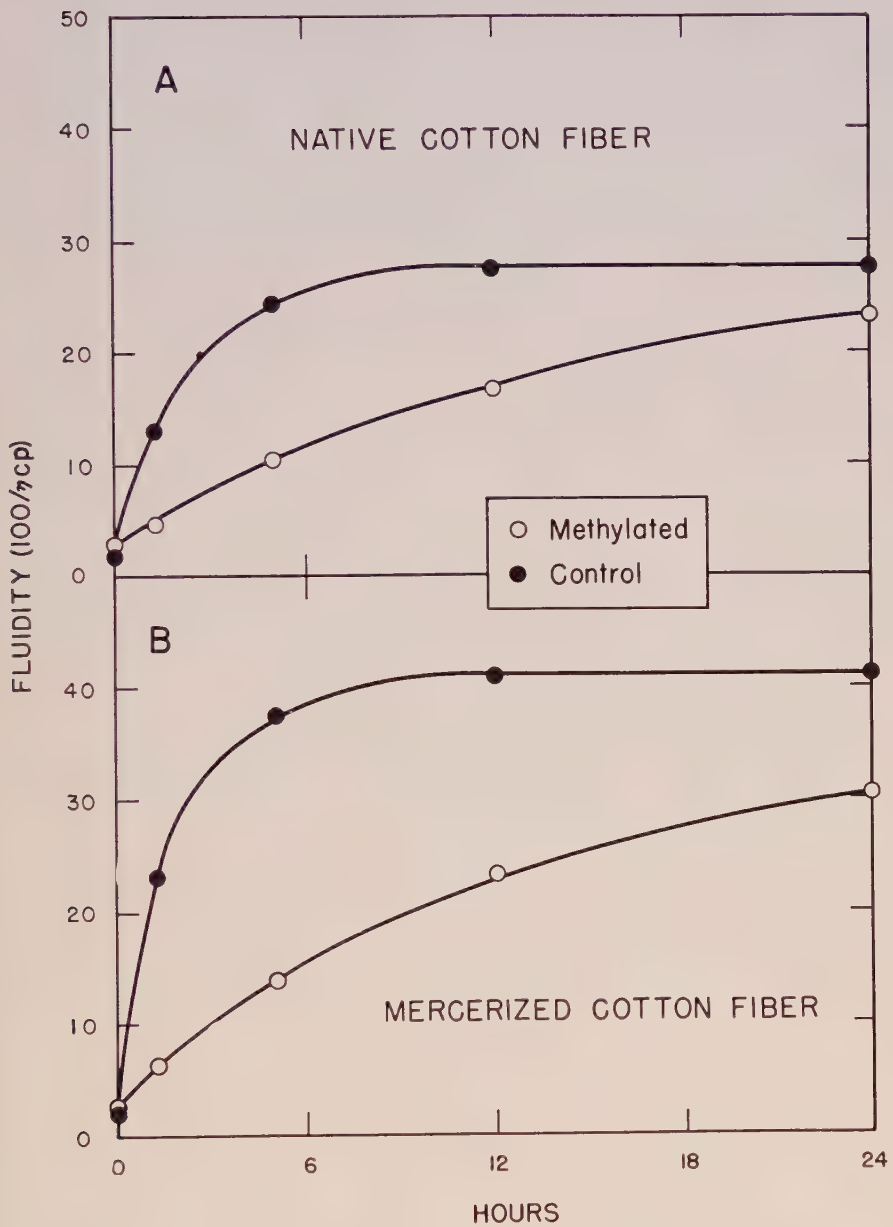

FIGURE 2. Cuprammonium fluidity of cotton fiber after oxidation with periodic acid. A. Alcohol-benzene, water extracted fiber before and after methylation (9.8 per cent methoxyl). B. Mercerized fiber before and after methylation (19.1 per cent methoxyl).

Sodium hypochlorite oxidation. A 0.05 N solution of sodium hypochlorite containing 1 per cent phosphate buffer was adjusted to pH 8.33. Samples of the unmethylated and partly methylated native fibers were treated with 100 parts of this solution for various lengths of time at 25° C. As is shown in Table IV none of the samples exhibited increased protection against the action of this reagent. Further work will be required to discover whether the methyl substituent is ineffective against hypochlorite because the latter attacks unsubstituted positions or because it is able to destroy a position even though the position bears a methoxyl group.

TABLE IV

OXIDATION OF UNSUBSTITUTED AND PARTLY METHYLATED NATIVE COTTON FIBER WITH ALKALINE HYPOCHLORITE

Time of oxidation, hours	Viscosity (ηcp) after oxidation with 100 parts 0.05 N hypochlorite at 25°, pH 8.33		
	Unsubstituted fiber	3.33% Methoxyl	9.82% Methoxyl
0	51.4	53.0	33.7
0.5	14.2	17.5	12.8
1	12.3	13.1	9.7
2	8.5	9.1	7.4
4	6.1	6.0	5.5
8	4.5	4.3	4.5
20	3.0	2.7	3.3

SUMMARY

1. Raw cotton or alcohol-benzene extracted cotton fiber reacts with a measurable amount of periodic acid before loss of viscosity is noted. Mercerized or water extracted fiber suffered a loss of viscosity with the smallest amounts of reagent which were tested.

2. Methylation of native cotton fiber (with diazomethane) to the extent of 9.8 per cent, or mercerized fiber to the extent of 19.1 per cent gives greatly increased resistance against oxidation by periodic acid.

3. Methylated cotton fiber which resists oxidation by periodic acid does not show enhanced resistance against the action of sodium hypochlorite.

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CHEMICAL REACTIVITY OF COTTON FIBER AS RELATED TO TYPE OF X-RAY DIFFRACTION PATTERN[1]

Richard E. Reeves and Wayne A. Sisson

In addition to the well known native and mercerized patterns, cotton fibers can be treated so that they will yield a mixed diagram containing both native and mercerized X-ray diffraction patterns superimposed. Such fibers have been called by Sisson and Saner (3) "partially mercerized." It is believed that these fibers contain crystalline areas which are in part native and in part in the mercerized or hydrate state.

The present experiments compare the change in a certain type of chemical reactivity with changes in the crystalline state of cellulose as shown by the X-ray diffraction pattern of cotton fibers. Reeves and Thompson (2) noted that native and mercerized fibers exhibit marked differences in extent of methylation by cold ethereal diazomethane. To find how closely this reactivity is related to the type of X-ray diagram, a number of native, partially mercerized, and mercerized samples were methylated all under similar conditions and the extent of substitution in each determined. Some of the partially mercerized fibers were prepared directly from raw fiber, while others were obtained from mercerized fiber by causing it to revert, in part, to the native state. Comparison of X-ray diagrams (before methylation) with extent of methylation shows a high correlation between this particular reactivity and crystalline structure.

MATERIALS AND METHODS

All alkali treatments were carried out by immersing, for ten minutes, mature raw cotton fibers (*Gossypium hirsutum* L. Super Seven variety, Strain 4) without tension in approximately 100 parts of sodium hydroxide solution which had previously been brought to the desired temperature in a water bath or freezing mixture. Upon removal from the caustic solution all samples were washed for 30 minutes at the same temperature, unless otherwise noted.

The air-dried fibers were tied in bundles approximately 1.0 mm. in diameter and exposed over the pinhole of the X-ray apparatus from 15 to 45 minutes. X-ray diagrams were made with unfiltered copper radiation ($K_\alpha = 1.54$ Å) produced in a Phillips Metalix tube operating at 28 kilovolts and 25 milliamperes. The X-ray beam was defined through a pinhole sys-

[1] A contribution from the former Cellulose Department of the Chemical Foundation, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York.

tem using 0.6 mm. pinholes placed 10 cm. apart. The sample to plate distance was 2 cm.

Diazomethane was prepared from nitrosomethyl urea according to the directions of Arndt in Organic Syntheses (1). All methylations were conducted simultaneously using 0.2 g. of air-dried fibers. Each sample was placed in a test tube together with 20 cc. of a solution of diazomethane in ether. The tubes were closed with stoppers bearing capillary outlets and stored in the refrigerator at 0° to 5° C. for eight days. The diazomethane solution was then replaced with a freshly prepared solution and the re-

TABLE I

TYPE OF X-RAY DIFFRACTION PATTERN AND EXTENT OF METHYLATION OF COTTON FIBERS AFTER VARIOUS TREATMENTS WITH SODIUM HYDROXIDE SOLUTIONS

Treatment		X-ray pattern, type*	Methoxyl, %
Conc. NaOH, %	Temperature, °C.		
9	18	N	6.3
9	−10 (washed at 0°)	M	12.6
12.5	60	N	5.6
12.5	18	P	9.8
12.5	10	P (almost M)	10.7
12.5	−10 (washed at 0°)	M	12.1
18	60	P	8.7
18	18	M	11.0
25	60	P	9.2
25	18	M	10.9
40	60	P	9.8
40	35	M	11.1
40	0	N	7.9

* N = native; P = partially mercerized; M = fully mercerized.

action allowed to proceed for another week. The concentrations of the first and second diazomethane solutions were 0.22 and 0.57 Molar, respectively. At the end of the second treatment the fibers were washed with ether containing acetic acid, then with alcohol and water. All analytical values are reported on the basis of air-dried samples.

In preparing partially mercerized fiber by reversion, the samples taken directly from the mercerizing bath were thrown into boiling water, or washed free of alkali and placed in glycerol which was then heated to 290° for 30 minutes.

RESULTS

In Table I are listed various treatments given to raw cotton fibers, together with the type of X-ray pattern produced and the amount of methoxyl taken up by the treated fibers.

It is apparent that native fibers took up the least methoxyl, with partially mercerized fibers intermediate and fully mercerized fibers becoming most substituted.

In Table II, fully mercerized fibers caused to revert more or less completely to the native state are examined, and the change in type of pattern and extent of methylation is shown. These two properties are again seen to be closely related.

DISCUSSION

Sisson and Saner (3) have published a diagram showing the type of X-ray pattern produced in cotton fibers by treatment with concentrations

TABLE II

X-RAY DIFFRACTION PATTERN AND EXTENT OF METHYLATION OF MERCERIZED AND PARTIALLY REVERTED COTTON FIBERS

Mercerization		Subsequent treatment	X-ray pattern, type*	Methoxyl, %
NaOH, %	Temperature, °C.			
25	18	Washed at 18°	M	10.9
25	18	Boiling water, 1/2 hr.	P	9.3
18	18	Washed at 18°	M	11.0
18	18	Boiling water, 1/2 hr.	P	9.2
18	18	Washed at 18°, placed in glycerol at 290° for 1/2 hr.	P	7.3
18	60	Washed at 60°	P	8.7
18	60	Washed at 60°, placed in glycerol at 290° for 1/2 hr.	P (almost N)	6.3
12.5	−10	Washed at 0°	M	12.1
12.5	−10	Boiling water, 1/2 hr.	P	9.9
12.5	−10	Washed at 0°, placed in glycerol at 290° for 1/2 hr.	P	7.4

* N = native; P = partially mercerized; M = fully mercerized.

of sodium hydroxide up to 50 per cent at temperatures from −20° to 100° C. In Figure 1 this diagram is reproduced with the results of the present experiments superimposed in the form of circles centered at the concentrations and temperatures employed. A number within each circle indicates the per cent of methoxyl taken up by the treated fibers. Presented in this manner, it is possible to appreciate the fact, recognized by Sisson and Saner, that there is a gradual transition between the areas of native and fully mercerized X-ray diagrams.

The light area to the right in Figure 1, centered roughly at 5° and 40 per cent sodium hydroxide solution, is interesting because it has been shown that these conditions do not produce mercerized or partially mercerized X-ray patterns. A sample of fiber from this area (last sample in Table I) on methylation was slightly more reactive than other native

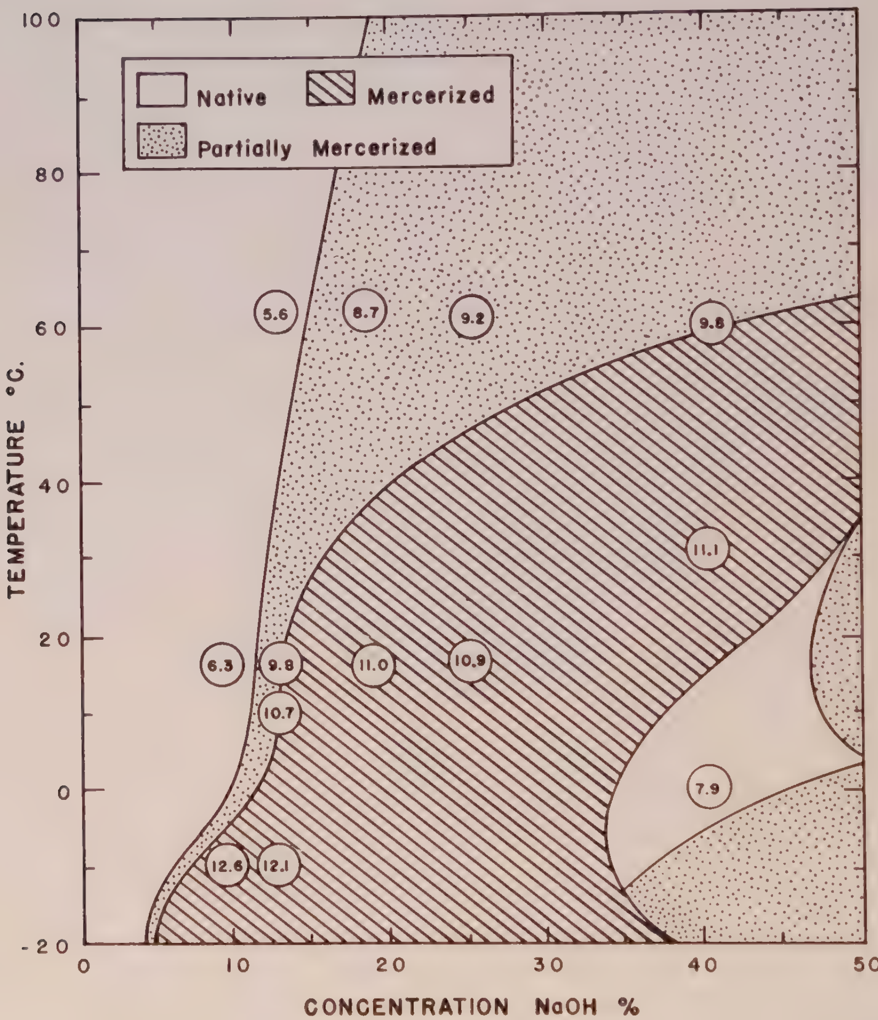

FIGURE 1. The reactivity of various native, partially mercerized, and mercerized cotton fibers. The numbers within the circles show the amount of methoxyl (in per cent) taken up following various alkaline treatments.

fibers, but clearly less reactive than samples from the mercerized and partially mercerized areas.

The results in Table II confirm the conclusion, drawn from X-ray work, that the hot glycerol treatment brings about more extensive reversion of mercerized fiber than the boiling water treatment. The methylation data indicate that in glycerol-treated fiber the reversion might be almost complete.

SUMMARY

The reactivity of native, partially mercerized, and mercerized cotton fibers with ethereal diazomethane was investigated. The extent of methylation was found to be lowest for native, intermediate for partially mercerized, and highest for fully mercerized cotton fibers. In these experiments change in reactivity was found to parallel change in the type of X-ray diffraction pattern obtained from the fibers.

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BIOLOGY OF THE HOLLY LEAF MINER

Albert Hartzell

The holly leaf miner (*Phytomyza ilicicola* Loew) is the most serious insect enemy of holly (*Ilex opaca* Ait.) in eastern United States (15). The presence of this species in injurious numbers at Yonkers, New York, and vicinity prompted a study of its life history. The chief injury is caused by the larvae mining the leaves, but punctures by the ovipositor in the leaves, for the purpose of egg laying and for feeding, also cause severe injury. Mined leaves present an unsightly appearance because of their yellowish-brown color which greatly lessens the value of the tree for ornamental purposes. As high as 80 per cent of the leaves may be disfigured by mines and the plant thereby weakened due to loss of chlorophyll from the destruction of extensive cell areas. Partial defoliation occurs following severe injury by the leaf miner especially during periods of drought. The control of the holly leaf miner is discussed in another paper (17). The present paper will be restricted, therefore, to a description of the insect, of which most of the early stages are here both described and illustrated for the first time, and to an account of the life history and habits of this interesting species of fly. The work has extended over a period of four years. While certain details of the life history and habits need further investigation, it is believed that the essential features of the bionomics of this species are as stated here.

The holly leaf miner is indigenous to North America and was first described by Loew (21) in 1863 as *Phytomyza ilicis*, but later the name was changed by him to *Phytomyza ilicicola* because the name *ilicis* had already been appropriated by Curtis for the European holly leaf miner. The European species occurs in England, France, Germany, and Holland on *Ilex aquifolium* L. (5). This species of leaf miner was accidentally introduced on the Pacific Coast of North America where extensive plantings of *Ilex aquifolium* are found in Oregon, Washington, and British Columbia (8). For a discussion of the synonymy of the two species of *Phytomyza* the reader is referred to a recent article by Cameron (5). Further taxonomic study may change the present conception regarding the species of this genus (23).

DISTRIBUTION

Phytomyza ilicicola is widely distributed throughout the Upper Austral, Lower Austral, and Transition life zones in North America. This species was reported in 1921 from Syosset, Long Island, New York, by Felt (10) and from Westchester County, New York, by Blauvelt (1) in 1937. The

insect also occurs in Massachusetts (14, 20), Connecticut (4, 12), New Jersey (20), Maryland (7, 20), District of Columbia (20, 21), Virginia (12, 25), North Carolina (20), Alabama (20), and Ohio (19). It has been intercepted in nursery stock shipped to the Pacific Coast but, according to Essig (9), has established itself at points ranging from California to Alaska. *Phytomyza ilicis* has been reported as established in several localities along the Atlantic Coast (3, 11, 13, 26, 27), but has not become a serious pest of holly due largely to the fact that it does not attack *Ilex opaca* which is the principal species of holly grown in eastern United States. In reviewing the literature it is difficult to determine in every instance whether a given locality record refers to this species because the name has been confused with *Phytomyza ilicis*.

HOST PLANTS

The holly leaf miner confines its attacks chiefly to *Ilex opaca*. It has been found occasionally on *Ilex cornuta* Lindl. and on *Ilex aquifolium* although it is not a serious pest of these two species. It has been reported also on *Ilex glabra* (19), and on *Ilex verticillata* var. *tenuifolia* (20). According to Cameron (5), *Phytomyza ilicis* confines its attacks entirely to the European species of holly (*Ilex aquifolium*).

DESCRIPTION OF THE INSECT

ADULT

The adult (Fig. 1 A) is a small dark colored fly about 2.5 mm. in length The first and second segments of the antenna are brown, the third is black, and the arista at most is 2.25 times as long as the third antennal segment. The thorax and femora are dusty gray. There is a yellow incisure present on the last abdominal segment of the female. This contrasts with *Phytomyza ilicis* in which the antennae are entirely black, the arista three times as long as the third antennal segment, and the thorax and femora dark. *Phytomyza ilicis* also lacks the yellow incisure in the last abdominal segment of the female (5).

EGG

The eggs (Fig. 1 B) are very small and difficult to find as they are laid beneath the epidermis of the holly leaf. Their presence is indicated by tiny green blisters (Fig. 1 E) on the lower side of the leaves. The eggs are laid in leaves of current growth. The leaf tissue at this time is soft and easily punctured by the ovipositor. The blisters are scattered widely over the leaf surface but many are located near the tip close to the midrib. These green blisters often are slightly discolored at the apical end. The egg is pure white, tapering at both ends, slightly blunted at one end; length 0.25 mm.

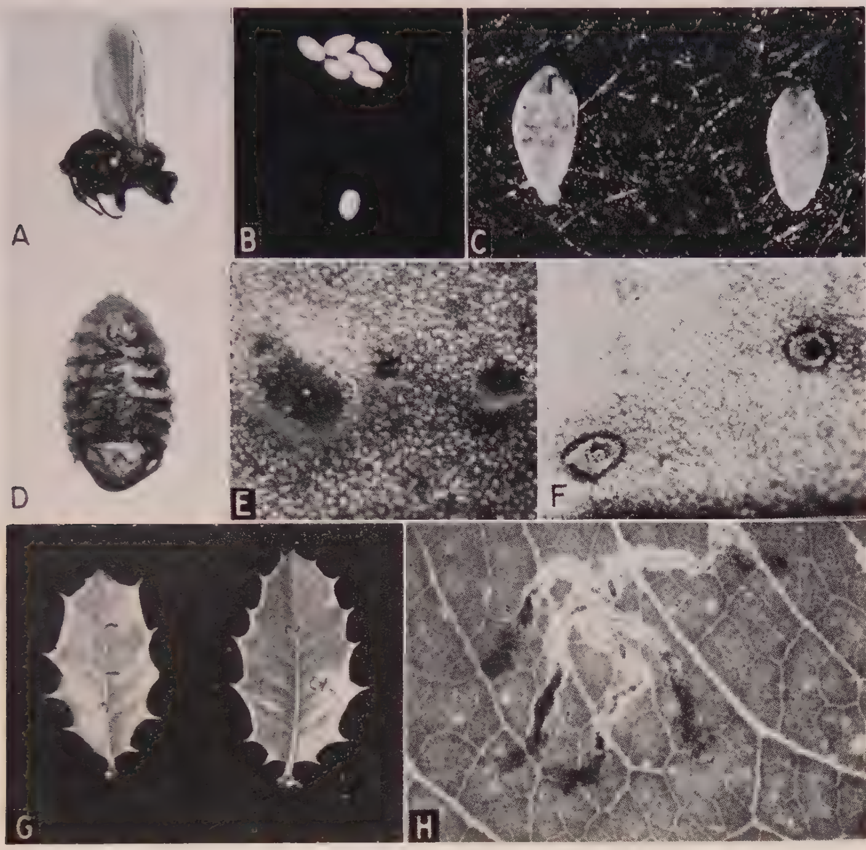

FIGURE 1. The holly leaf miner, *Phytomyza ilicicola*. A. Adult, ×10. B. Eggs, ×18. C. Larvae, ×12. Left, ventral view of larva; right, dorsal view. D. Pupa, ×12. E. Egg blister in holly leaf, ×18. F. Puncture made by ovipositor in holly leaf, ×18. G. Mines made by young larvae, ×0.7. H. Mines made by mature larvae, ×3.

LARVA

The larva (Fig. 1 C) is pale yellow and tapers from the anterior to the posterior end. The anterior spiracles of both *Phytomyza ilicicola* and *Phytomyza ilicis* tend to be approximated at the base. They project rather prominently (16, p. 66, 76).

First instar. Pyriform pale lemon yellow larva with smooth glistening integument. Head retracted into the thorax with only the fore-end exposed. The three oral hooks are dark brown. Malpighian tubes are dark brown and visible through the integument. The two oral spiracles ventral, dark brown; length 0.37 mm.

Second instar. Lemon yellow. Segments plainly visible. Oral hooks and malpighian tubes dark brown but more plainly visible through the integu-

ment than in first instar larva. Two oral spiracles dark brown, and very conspicuously elongated; length 0.9 mm.

Third instar. Resembling the second instar but much larger; length 1.5 mm.

PUPA

Oval in shape (Fig. 1 D) tapering uniformly to a blunt point at each end; segmented. Pale reddish-brown in color. Dorsal and ventral sides flattened; length 2 mm.

LIFE HISTORY

METHODS

Adult leaf miners were collected and transferred by means of a pneumatic insect collector. For rearing insects in the greenhouse or in an out-of-doors shelter several different kinds of cages were used depending upon the object to be attained. In caging small holly trees, lantern globe cages with the tops covered with wire screen (50 meshes per linear inch) were used. The trees were in 4-inch pots resting on glass plates. When larger trees were used cages sufficiently spacious ($66 \times 36 \times 24$ inches) to accommodate a dozen trees were employed. The cages were tightly constructed with wooden frames and floors. The sides were made of panes of glass while the ends and tops were covered with wire screen (50 meshes per linear inch). The greenhouse in which these cages were located was maintained at 70° F. at night. In the out-of-doors shelter the temperature averaged about 10° F. lower than in the greenhouse. Cheesecloth cages with wooden frames of approximately the same size as the above also were used in the out-of-doors shelter.

Cages made by cementing together strips of cheesecloth and cellophane were used in confining adults on branches or on individual leaves.

It was not found feasible to obtain measurements of individual larvae from the time they hatched until they pupated owing to the fact that larvae that have been disturbed in the mines usually perish. Ten larvae dissected from mines selected at random were collected and measured on the same day. The average length computed from the ten measurements was used as the average larval length for a given date. The average increase in length of larvae is shown graphically in Figure 2 as correlated with the average length of mines.

LONGEVITY OF ADULTS

The number of days that the adults were kept in captivity ranged from one to six. The females averaged three days in captivity when placed on holly leaves, while the males averaged only two days. Adults of both sexes

lived less than one day without food. The earliest date on which the adults were observed in the field at Yonkers, New York, was May 10. The latest record was June 27.

OVIPOSITION

The period of oviposition of this species is believed to be relatively short as indicated by the longevity of the females. Holly leaves in the greenhouse exposed to fertile females on May 28 showed faint traces of mines on June 1. This would indicate an incubation period of not more than 4 days. Under field conditions this period would be somewhat longer.

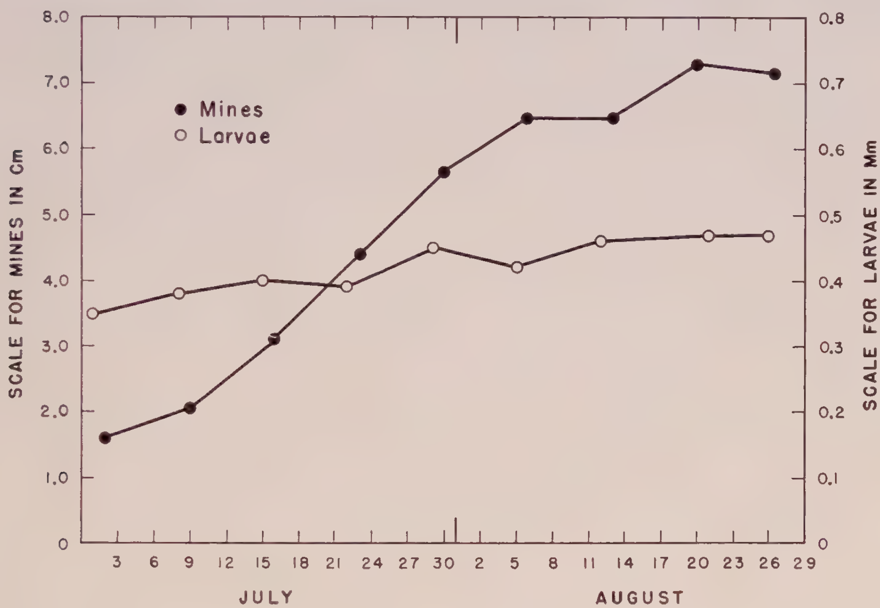

FIGURE 2. Comparison of average larval length with average length of mine, 1942.

LENGTH OF LARVAL PERIOD

The larvae were found from June 16 to April 10 of the following year. Weekly measurements were made of larvae throughout the spring, summer, and autumn for the detection of individuals that had moulted. The first instar was noted June 16. The first individual of the second instar was observed November 10, while the first individual of the third instar was noted on December 27. The latest date in the spring that a larva was observed was April 15. By April 29 all had pupated. Langford and Cory (20) reported that 64.5 per cent of the larvae had pupated at College Park, Maryland, on April 3 and 90.9 per cent by April 20.

LENGTH OF THE PUPAL PERIOD

Holly leaves examined April 15 showed mostly pupae present in the mines, with an occasional larva. Pupae were present in decreasing numbers until the middle of June. Willey (12) reports all individuals examined on March 14 at Richmond, Virginia, as being in the pupal stage.

TOTAL LIFE CYCLE

The adults were found over a period of about six weeks (Fig. 3). Oviposition occurs during May and June. Eggs were found in the field from the latter half of May until the first week in July. The incubation period was found to be four days. The larval period averages about nine or ten

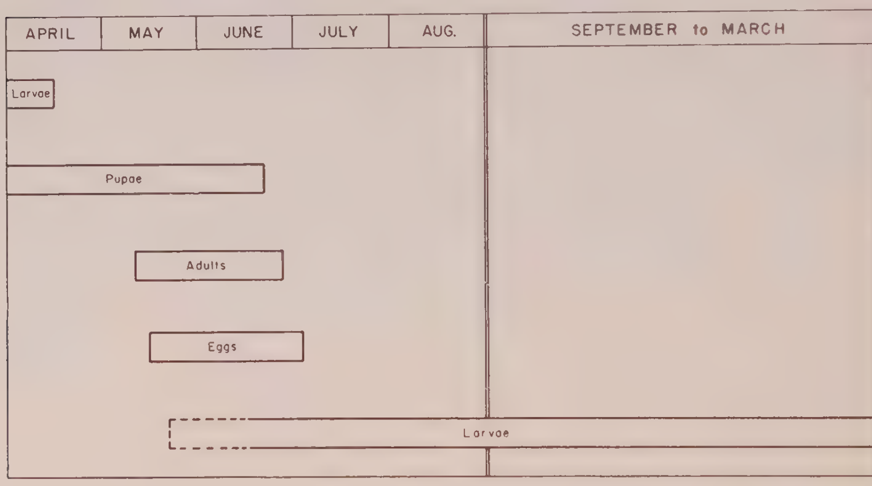

FIGURE 3. Life cycle of the holly leaf miner, *Phytomyza ilicicola*.

months. The pupae were found in the field from the first week in April until the middle of June.

To summarize, the overwintering larvae pupate almost with the first growth of holly in the spring. The adults emerge and lay their eggs in the leaves as they unfold from the buds. The larvae mine the leaves throughout the remainder of the growing season and pupate in the spring. The comparatively long time for the insect to reach maturity precludes the possibility of its being two-brooded in the latitude of Yonkers, New York.

HABITS OF THE INSECT

FEEDING

It is well known that species of *Phytomyza* puncture leaves with the ovipositor for the purpose of feeding. Eggs are not deposited in these punctures or, if deposited, the eggs never hatch. Cohen (6), for example,

reports finding only one larva to 25 punctures made by *Phytomyza atricornis* Mg., a related species of leaf miner on chrysanthemum. He points out the fact that male flies are dependent on the female feeding punctures and will starve when caged on unpunctured leaves.

The feeding habits of *Phytomyza ilicicola* females were observed on several occasions by caging adults on young holly plants in bell jars in the laboratory. The female inserted its ovipositor in the leaf followed by three or four back and forth movements during which the body was arched indicating an exerted strain to puncture. Just as soon as the ovipositor was removed the fly quickly backed away and lowered its mouth parts to the puncture and began feeding. The flies appear to select positions near the margin of the leaf for insertion of the ovipositor. Punctures are mainly interveinal, arranged in groups, and are confined almost entirely to newly expanded leaves. In fact there is a very marked gradient in the number of punctures from the youngest to the oldest leaves.

Male flies placed on tender foliage produced no punctures and apparently died of starvation. When male and female flies were caged together on holly foliage it was noted that the males lived longer than when caged alone on holly leaves. The results indicate that the feeding habits of *Phytomyza ilicicola* are similar to *Phytomyza atricornis*.

Female flies when given a choice between *Ilex opaca* and *Ilex aquifolium* plants showed a marked preference for the former species. Punctures of *Ilex opaca* leaves ranged from a few to 50 per leaf while leaves of *Ilex aquifolium* showed no punctures. When females were caged on *Ilex aquifolium* leaves, however, a few scattered punctures were made.

The larva when feeding lies on its side and mows down the plant cells with its mouth hooks. The destruction of plant tissue by the larva is confined to the mine which is located between the upper and lower epidermis of the leaf.

HABITS OF THE ADULTS

In collecting adults of *Phytomyza ilicicola* it was noticed that the males are very active. They take to flight at the slightest disturbance. The males frequent both the upper and lower surface of the mature holly leaves. The female flies are tamer and somewhat more sluggish than the males. The females are in general found close by on the folds of the immature holly leaves. Adults were observed mating May 21.

If holly leaves are carefully examined pits will often be noticed on both the upper and lower surfaces of the leaves. The pits first appear when the leaves are young but they are found on fully developed leaves with no increase in numbers. They were first thought to be caused by castigation, i.e., by the spines of the older leaves pricking the young leaves at time of high wind. Holly leaves which are not infested with flies do not show pits on the leaves. Miall and Taylor (22) found that *Phytomyza ilicis* produces

similar punctures on holly. In the present investigation female flies of *Phytomyza ilicicola* were observed piercing the leaves with the ovipositor for the purpose of feeding. It was possible to produce pits by caging adult females on holly leaves free from wind currents (Fig. 1 F). It requires from one to four females per plant to produce several dozen punctures. The age of the leaf and its relative succulency make the difference between few or many punctures. The pits are the feeding punctures caused by the insect as discussed in the previous section.

If during unfolding of the bud the leaf margin is injured by means of repeated punctures the cells of the leaf tissue immediately adjacent to the punctures are killed, causing distortion of the leaf. Boyce and Korsmeier (2) have shown that a similar type of distortion is produced on lemon leaves which develop from buds infested with the citrus bud mite (*Eriphyes sheldoni* Ewing).

HABITS OF THE LARVAE

The egg punctures are found on the lower surface of the leaf, but the larva on hatching soon passes to the palisade layer of the mesophyl and mines just beneath the upper epidermis. The mines are linear, irregular, and serpentine (Fig. 1 G). Usually there is one larva per mine. Smaller mines anastomose with larger mines (Fig. 1 H). Such mines may envelop a whole leaf and may contain three or more larvae. The mine is gradually widened and later deepened to form a cavity in which pupation takes place. Just before pupation the larva prepares an exit by cutting a circular opening which is covered with a layer of leaf epidermis through which the spiracles are extended. The larva of the European holly leaf miner (*Phytomyza ilicis*), according to Miall and Taylor (22), enters the midrib on hatching and tunnels the midrib for about two months during which time it remains hidden from view and gives little or no indication of its presence. In September, October, or November it leaves the midrib and enters the blade of the leaf and produces a blotch-like mine which is usually the first indication of infestation. This habit was not observed with *Phytomyza ilicicola*. Many of the mines of this species are in the blade of the leaf far removed from the midrib and in positions that suggest little or no relationship with the midrib as to their origin. Furthermore, *Phytomyza ilicicola* typically produces linear serpentine mines rather than blotch-like mines.

SEASONAL HISTORY

CLIMATIC FACTORS

The time of emergence of the adults appears to be dependent on the weather conditions during early spring. In a normal month such as May, 1938, with a mean temperature of 59.8° F., and 3.38 inches of rainfall (18, 24), the first flies were observed in the field on May 16. During a late

season such as 1940, with the mean temperature of 60.6° F., and 7.61 inches precipitation in May (18, 24), the flies were not observed until June 3. The seasons of 1939, 1941, and 1942 were relatively dry and warm during the early spring. The first flies were observed in the field on May 10, May 14, and May 12, respectively. Since the adults lay their eggs shortly after emergence, oviposition, incubation, and the early development of the larvae are correspondingly advanced or retarded by weather conditions affecting the emergence of the adults.

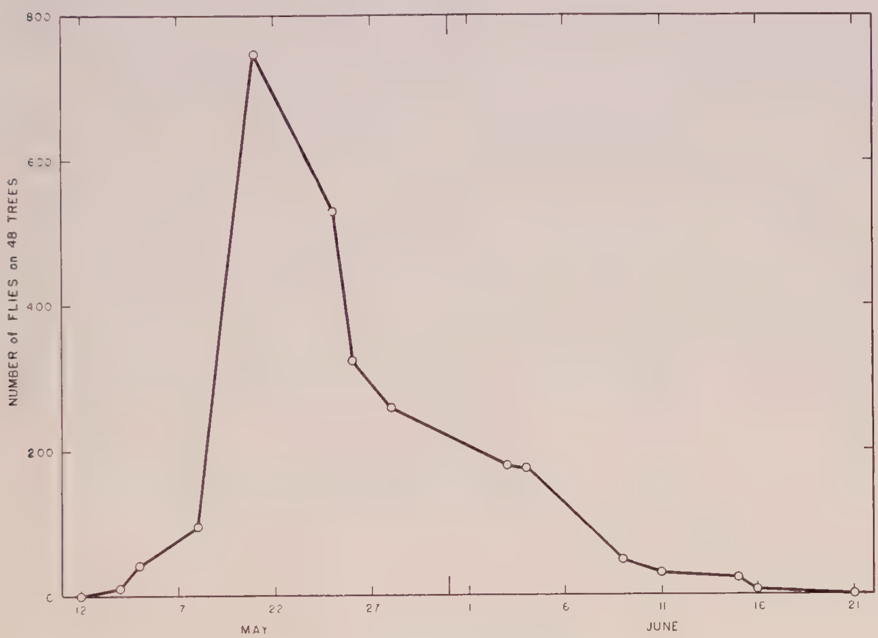

FIGURE 4. Population of adults, 1942.

HIBERNATION

The holly leaf miner winters in the mine usually as a third instar larva. Pupation takes place at the time of the first growth of the leaves in the spring, usually during the period from the first week in March until the last week in April.

POPULATION STUDIES

Population counts were made by observing adult flies on holly trees (*Ilex opaca*) nine years old in nursery rows. Counts were made on the basis of 48 trees distributed in four rows. As far as possible all counts were made by the same person at the same hour of the day. The interval between counts averaged three days. The results for 1942 are shown in Figure 4. The first flies, it will be observed, were seen on May 12, when two were

noted. There was a rapid rise in the number of adults observed until May 21, when the peak of the brood was reached with a count of 747 flies. After this date there was a gradual decrease until June 21 when the last flies of the season were observed.

Langford and Cory (20) report that most of the flies emerged between May 1 and May 20 at College Park, Maryland.

NATURAL ENEMIES

The writer observed an unidentified fungus parasite of the pupa. Several attempts to rear parasitic insects from infested leaves were unsuccessful. *Opius striativentris* Gahan, *Closterocerus tricinctus* Ashm., *Chrysocharis* sp., and *Horsimenus* sp. have been reported as parasitic on *Phytomyza ilicicola* by Underhill (25) and Langford and Cory (20). The first named species and *Sympiesis felti* Crawf. have been reported by Willey (28) as parasitic on *Phytomyza ilicis*.

SUMMARY

The holly leaf miner (*Phytomyza ilicicola* Loew) is indigenous to North America where it is found principally in the Atlantic and Pacific coastal regions. In eastern United States the range of the species extends from Massachusetts to Ohio and as far south as Alabama. In western North America it has been reported as established at points ranging from California to Alaska.

Ilex opaca Ait. is the principal host of *Phytomyza ilicicola*, but the insect occurs on several other species of holly including *Ilex aquifolium* L.

The primary injury of *Phytomyza ilicicola* is caused by the larvae mining the leaves of holly, but oviposition and feeding punctures in the leaves also cause injury.

Phytomyza ilicicola is single-brooded in the latitude of Yonkers, New York. The insect passes the winter in the mines usually as a larva. Pupation occurs with the first growth of the holly plant in the spring. The adults emerge in May and June and lay the eggs in the leaves. The incubation period is short, requiring only four or five days. The larval period extends from the middle of June to the latter part of March. The pupal period is about one month in length. The adults emerge the fore part of May.

Aside from an unidentified fungus attacking the pupa, no predacious or parasitic enemies of *Phytomyza ilicicola* were observed.

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CONTROL OF THE HOLLY LEAF MINER

Albert Hartzell, Donald L. Collins,[1] and
William E. Blauvelt[1]

In the experiments described by Hartzell and McKenna in 1941 (4) certain treatments which had been recommended in the literature (1, 2) for the control of the holly leaf miner (*Phytomyza ilicicola* Loew) did not give satisfactory results. Hartzell and McKenna reviewed previous work on the subject and presented additional evidence that proper timing is a critical factor in the success or failure of a spray. It should be noted that the control recommendations appearing in the literature are, for the most part, based on experiments of limited scope or on the work of a single season. All authors stress the importance of applying the sprays and dusts at a time determined by the presence of adult flies on the foliage. Since it is obvious that this time must vary with the weather and the location, and probably with unknown factors in the biology of the species, a separate study of the life history and habits of the flies was made (3). The experiments described in the present paper were designed to afford a comparison between several recommended control measures and the best of those obtained by Hartzell and McKenna, namely the nicotine sulphate and fish oil, and nicotine sulphate, fish oil, and lead arsenate formulae.

MATERIALS AND METHODS

Description of plots. Plots were laid out in a holly (*Ilex opaca* Ait.) planting nine years old grown from seed and from cuttings. Each treatment was replicated three times, once in each of three blocks. The basic plot unit was eight trees, but most of the plots actually contained only six or seven trees, which averaged about seven feet in height. The plot and block arrangements are shown in Figure 1.

That a sufficiently even normal distribution of adult flies occurred was shown by counts of the flies made on May 23, two days before the first sprays were applied to certain plots. In nine plots, including 60 trees, the numbers of flies that could be seen by the observer as he walked around each tree once were counted. The counts were made in the plots that were to be sprayed with tartar emetic and sugar and with lead arsenate, and in the checks. The counts, on a treatment basis, averaged 37, 29, and 44 flies per tree, respectively.

Evaluation of results. The effects of the treatments were judged from the relative numbers of mines found by examining the leaves in August. At

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this time the mines are sufficiently developed to permit counting, but the injury is not so severe as to cause the leaves to fall. In a dry season leaves that are extensively mined may drop off early in the autumn so that counts made after late September are inaccurate. Approximately 200 leaves each from three trees in each plot were examined. The procedure was to start at the top of the tree and count down 200 leaves, examining all the current season leaves except the very tender shoots of late summer growth, and recording all of the mines. The data are here expressed as the number of mines per 10 leaves.

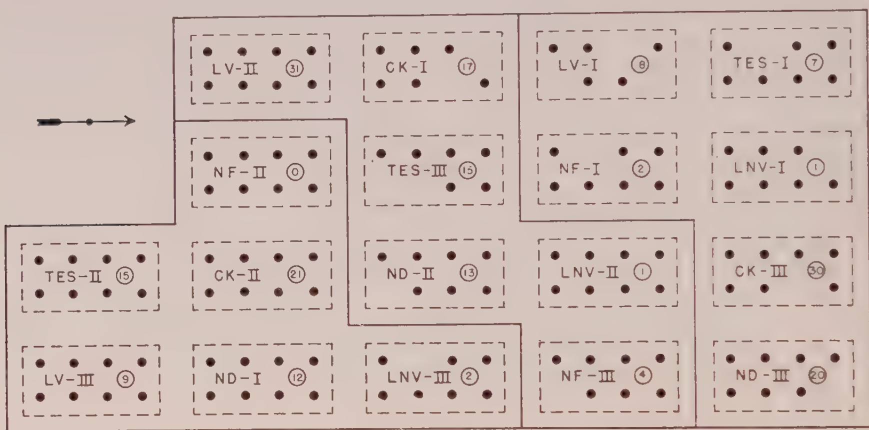

FIGURE 1. Plot arrangement for holly leaf miner spray experiments. Figures in large circles represent average numbers of mines per 10 leaves per plot, counted in August, 1942. (See Table I for spray formulae.)

Treatments. The sprays and dust used are described in Table I. The dust was applied with a rotary fan hand duster. The sprays were applied with a 50-gallon power sprayer at a pressure of approximately 300 pounds.

RESULTS

The results of the treatments, together with other pertinent data, are presented in Table I. It is evident that both the lead arsenate and nicotine sulphate combination, and nicotine sulphate with fish oil alone, gave adequate control. It is clear that neither one application of lead arsenate, two applications of nicotine-lime dust, nor two applications of tartar emetic and sugar were effective. It should be noted that during the two weeks following the single application of lead arsenate and the first application of tartar emetic and sugar, there were five days of rain, including at least one exceptionally severe storm. Since analysis showed the differences between the figures from the tartar emetic plots and those from the checks

to be almost significant, the tartar emetic sprays perhaps deserve further tests.

Two supplementary tests of the tartar emetic and lead arsenate formulae were carried on in order to run parallel experiments under conditions more closely approximating those under which the holly is generally used

TABLE I

RÉSUMÉ OF DATA ON SPRAYING FOR CONTROL OF HOLLY LEAF MINER

Formula	Applications, 1942	Plot No.	No. leaves counted	No. mines counted	No. mines per 10 leaves (Average per treatment)
Nicotine Lime dust 3% (ND)	May 21, 28	I II III	621 621 617	729 850 1244	15.2
Lead arsenate 4 lbs. Nic. sulph. 1 qt. Vatsol* 400 cc. Water to make 100 gals. (LNV)	May 14, 25 and June 9	I II III	624 603 613	54 75 117	1.3
Nic. sulph. 1 qt. Fish oil 2 qts. Water to make 100 gals. (NF)	May 14, 25 and June 9	I II III	651 651 606	136 31 246	2.2
Lead arsenate 4 lbs. Vatsol* 400 cc. Water to make 100 gals. (LV)	May 25	I II III	709 662 584	1207 2034 551	19.4
Tartar emetic 4 lbs. Sugar 6 lbs. Water to make 100 gals. (TES)	May 25 and June 10	I II III	634 671 643	499 992 1000	12.8
Check (Ck)		I II III	605 612 615	1023 1273 1821	22.5

* 10 per cent aqueous dioctyl ester of sodium sulphosuccinate, product of American Cyanamid and Chemical Corp., Bridgeville (Pittsburgh), Pa.

as an ornamental. One of these locations was on the Wallerstein estate in Harrison, New York, the other at the Raven Rock nurseries near Elmsford, New York. Only one application each of the tartar emetic and lead arsenate sprays was used.

At the Wallerstein estate there were two holly trees, set several hundred feet from one another among other trees and shrubs. They were vigorous trees, about 14 feet high. They were sprayed on May 16, using a

three-gallon compressed-air sprayer with a six-foot boom and adjustable nozzle. At this time only three adult flies were seen on both trees, although they were numerous in Yonkers the previous day. Tree *A* was sprayed with a mixture of lead arsenate and wheat flour in the proportion of 6 pounds of each in 100 gallons of water. Tree *B* was sprayed with tartar emetic and brown sugar at the rate of 6 pounds of each in 100 gallons of water. At the time of spraying, examinations of 500 older leaves from each tree showed an average of 4.5 mines per 10 leaves on tree *A* and 2.5 mines per 10 leaves on tree *B*. When the final examinations were made early in September tree *A* had 5.57 mines per 10 leaves and tree *B* had 0.28 mine per 10 leaves. It is questionable whether these data reflect a real increase and decrease, respectively, as compared to the initial infestation. However, had no treatment been applied, the figure would have been expected to have been higher in the fall, because many heavily mined leaves are lost each winter, thus lowering the spring counts. The fact that it was higher in the fall on the tree sprayed with lead arsenate may, nevertheless, indicate a lack of control, since on the tree sprayed with tartar emetic the count in the fall was lower than in the spring, indicating a possible effect from the spray. This interpretation is strengthened by the data from the main experiment.

The second supplementary experiment, involving seven trees in the Raven Rock nursery, failed to produce conclusive results. Two trees sprayed with lead arsenate showed a decrease in the fall and one showed an increase, while two trees sprayed with tartar emetic showed an increase and one showed a decrease.

DISCUSSION

An inspection of the curve of adult fly abundance for 1942 (3) with reference to the results of the spray operations affords interesting material for speculation. The first flies were noticed on May 12, after which there was a slow but steady increase in numbers through May 18, with a very sudden increase to a peak on May 21. The first sprays of the 3-spray series in the main planting were applied on May 14. The only sprays at the Wallerstein estate and Raven Rock nursery were applied on May 16 and 18, respectively. The latter sprays produced rather ambiguous results, but in general they were disappointing. The second sprays of the 3-spray series, the first spray of the 2-spray series of tartar emetic and sugar, and the only spray with lead arsenate and a spreader alone were applied in the main planting on May 25. This date was during the second part of the "peak" period of fly emergence. Thereafter the adult flies declined steadily in numbers until by June 9, when the last sprays were applied, the population was approaching its lowest level. From these comparisons it seems likely that one spray of any material applied several days before the peak would not

be satisfactory, nor would it be satisfactory if delayed more than a day or so after the peak. The spray applied on June 9 probably had little effect on the final results. It is probable that the first sprays of the 3-spray series were applied at the right time to have an effect on the entire pre-peak population of flies, that the second sprays were applied at the right time for the peak and declining populations, and that, therefore, the satisfactory results of both of these sprays reflect a cumulative effect of the two applications.

SUMMARY AND CONCLUSIONS

Considering the data from the spray experiments in relation to the seasonal history of the insect it is concluded that, with a moderate to heavy infestation one spray of any of the materials tried could not be expected, except by chance, to produce adequate control of the holly leaf miner, because of the difficulty in timing the spray. Three applications of nicotine sulphate and fish oil produced a good control, which was not appreciably improved by the addition of lead arsenate. It is probable that two applications would be sufficient in a normal year, if one is made two or three days before the peak, and the other two or three days after the peak of emergence of the adult flies.

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EFFECT OF MOISTURE FLUCTUATIONS ON THE VIABILITY OF SEEDS IN STORAGE

LELA V. BARTON

It has been known for a long time that the three most important environmental factors affecting the length of life of seeds under storage conditions are temperature, humidity, and gaseous exchange. A great deal of literature has appeared on this subject and no attempt will be made here to review this work. In a description of storage effects on some flower seeds, the present author reported in 1939 (1) that if containers in which dandelion seeds were stored were opened as often as every six months, it was necessary to reduce the moisture content to as low as 3.9 per cent at the beginning of the storage period in order to keep the seeds viable for three years, whereas seeds sealed in glass tubes which were not opened before the time of testing kept much better. This difference in life-span was attributed to fluctuation in moisture content in the seeds in containers which were opened for testing and then resealed. This interpretation was based on actual moisture determinations made on seeds subjected to the two types of storage conditions.

Subsequently a test was set up, the design of which included controlled fluctuation of humidity at constant temperature. The temperature used was 20° C. Desiccators with different relative humidities were used as storage chambers. A constant humidity was maintained in each desiccator by saturated salt solutions with an excess of the salt (2). Thus above saturated solutions of magnesium chloride, calcium nitrate, and sodium chloride relative humidities of approximately 35, 55, and 76 per cent respectively were obtained. The seeds were placed in cheesecloth bags in these desiccators. Fluctuations between 35 and 55 per cent relative humidities, between 35 and 76 per cent, and between 55 and 76 per cent at intervals of 2, 4, and 8 weeks were made by transferring the bags containing the seeds from one desiccator to another. Certain lots were left at each of the constant humidities for the entire storage period as controls. The seeds used were onion (*Allium cepa* L.), dandelion (*Taraxacum officinale* Weber), eggplant (*Solanum melongena* L.), and tomato (*Lycopersicon esculentum* Mill.), the first two because they are very sensitive to adverse storage conditions and the second two because they are relatively resistant to the same conditions. Viability tests were made on moist filter paper in ovens at controlled temperature, except dandelion which was germinated in the laboratory. Germination temperatures used were 25° C.

for onion and tomato and a daily alternation of 20° to 30° C. for eggplant. Both viability tests and moisture determinations were made each two weeks up to 64 weeks of storage or until the particular lot of seeds had lost their power to germinate. For moisture determinations seeds were dried in a vacuum oven at 80° C. for 48 hours.

RESULTS

1941 TESTS

Moisture Determinations

The moisture contents attained by the various seeds under different humidities in storage are shown in Table I. These figures represent averages of all of the determinations made under a given set of conditions at various

TABLE I

AVERAGE MOISTURE CONTENT (% OF DRY WT.) ATTAINED AT EACH HUMIDITY, CONSTANT AND ALTERNATING

Alternation		Dandelion		Eggplant		Tomato		Onion	
Time	% Relative humidity								
None	35	7.0		7.3		7.7		8.6	
	55	9.4		10.0		10.1		11.4	
	76	12.2		13.0		12.9		14.9	
		Lower humidity	Higher humidity	Lower humidity	Higher humidity	Lower humidity	Higher humidity	Lower humidity	Higher humidity
2-Weekly	35–55	7.8	9.3	8.2	9.6	8.4	9.5	9.4	11.0
	35–76	8.0	11.6	8.3	12.5	8.6	12.1	9.7	14.4
	55–76	9.9	12.3	10.6	12.7	10.7	12.7	12.4	14.4
4-Weekly	35–55	7.3	8.7	7.7	9.3	8.0	9.4	8.9	10.7
	35–76	7.3	11.6	7.8	12.3	8.1	11.9	9.0	14.4
	55–76	9.6	11.6	10.5	12.5	10.6	12.4	12.1	14.8
8-Weekly	35–55	7.2	8.8	7.5	9.3	7.9	9.4	8.7	10.7
	35–76	7.2	11.8	7.6	12.4	7.9	12.2	8.6	14.7
	55–76	9.3	12.1	10.2	12.6	10.2	12.7	11.7	15.5

times after storage. For example, the moisture in seeds kept at constant humidities was determined at intervals of 2 weeks up to 36 to 52 weeks so that the figures in the table are averages of 18 to 26 separate determinations. Similarly moisture determinations were made at the time of each transfer from one humidity chamber to another in order to determine just how much fluctuation in moisture within the seed resulted from each treatment. Repeated determinations were made so that any unusual circumstance such as wetting of seeds with solution, condensation of moisture

on the sides of the desiccators, or unsaturated solutions and hence change of humidity could be detected. These moisture tests revealed a uniformity of water content for any one species of seed under a given set of conditions. The amount of moisture absorbed varied with the kind of seed. Dandelion

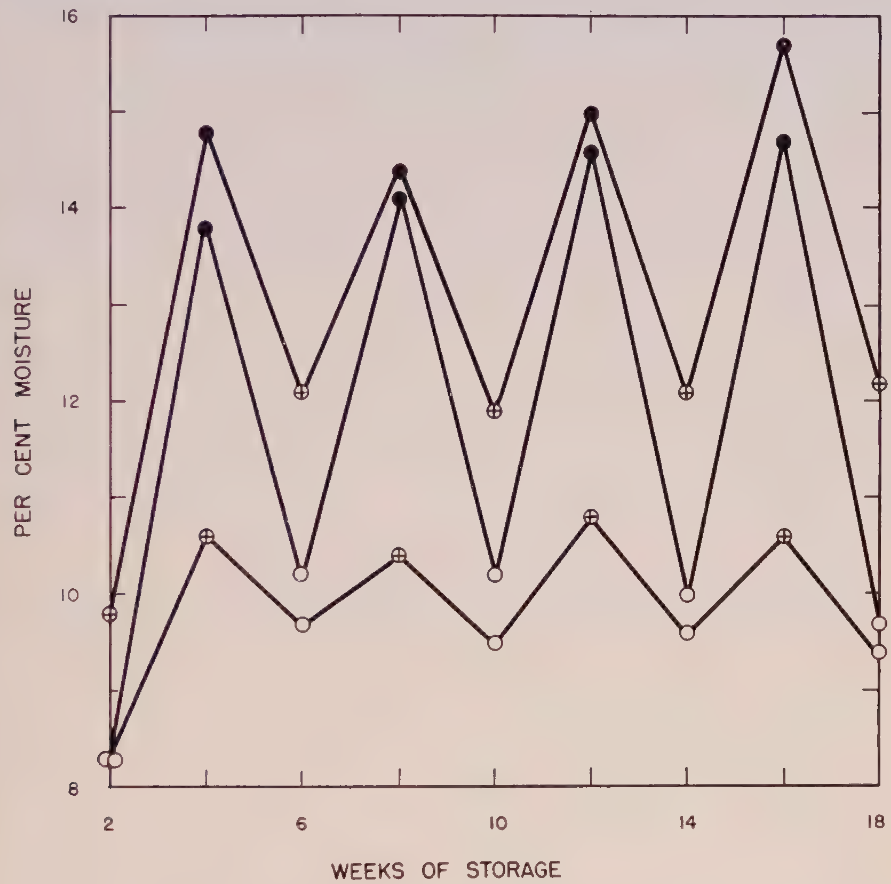

FIGURE 1. Fluctuation in moisture content of onion seeds when alternated between two different relative humidities. Per cent moisture after two weeks at 35, 55, and 76 per cent relative humidities indicated by open circles, circles with crosses, and solid circles respectively.

absorbed least and onion most under any given set of conditions. Eggplant and tomato seeds absorbed approximately the same amounts of water under a given set of conditions.

It will be noted that, in general, when alternation from one humidity to another was effected the variation of the moisture content of the seeds tended to be the same as that indicated by the determinations made at

constant humidity storage. In some cases, especially if the alternations were made every two weeks, variations in moisture content of the seeds was somewhat less than that indicated by the determinations made at constant-humidity storage. Seeds transferred from a high to a low humidity failed to reach the low moisture expected before they were transferred again to a higher humidity, where again they failed to develop the maximum moisture content to be expected (Table I and Fig. 1).

Onion seeds held at constant relative humidities of 35, 55, or 76 per cent acquired moisture contents which averaged 8.6, 11.4, and 14.9 per cent and remained uniform throughout the test period. Onion seeds alternated between 35 and 55 per cent relative humidity attained an average of 9.4 per cent moisture after two weeks at 35 per cent and 11.0 per cent after two weeks at 55 per cent, so that the average variation in moisture content was 1.6 per cent every two weeks. Similarly the average variation when the seeds were changed from 35 to 76 or from 55 to 76 per cent every two weeks was 4.7 and 2.0 per cent respectively. This fluctuation in moisture can be seen in Figure 1, where the actual moisture contents as determined by weighing samples of onion seeds after each two weeks of storage are plotted. The data for the first eighteen weeks of 2-weekly alternations were used in these graphs. Similar variations were obtained for 4-weekly and 8-weekly alternations.

Viability Tests

Some of the data obtained from viability tests made at intervals of 2 weeks from the beginning up to 52 weeks are shown in Table II. Only onion results are presented here since dandelion seeds behaved in a similar manner and since neither eggplant nor tomato seeds showed any reduction in viability under any of the storage conditions except a constant relative humidity of 76 per cent up to 64 weeks at which time the experiment was terminated. The resistance of these seeds to unfavorable storage conditions has been reported before. It will be seen at once that a constant relative humidity of 35 per cent permitted retention of viability for one year at 20° C., with the percentage germination only slightly reduced at the end of that period. A reference to Table I will show that the moisture content of onion seeds at this relative humidity was approximately 9 per cent. At 55 per cent relative humidity at which onion seeds absorb about 11 per cent moisture, serious deterioration occurred after 20 weeks of storage. At 76 per cent relative humidity considerable loss in germinative power was evident after 8 weeks of storage and after 12 weeks at this humidity where the moisture content of the seeds was about 15 per cent, onion seeds yielded only 23 per cent germination as compared with 96 per cent at the beginning of the special storage period.

These comparative effects of low, medium, and high humidity storage chambers were to be expected since previous work has demonstrated simi-

lar onion seed characteristics. But the question remains as to whether alternation of humidities in the storage chamber and hence fluctuation of moisture content within the seed itself is more deleterious than a constant high moisture content. At first it appeared that the answer to this question was negative. Seeds which had 2-weekly, 4-weekly, or 8-weekly alternations from 35 to 55 per cent relative humidity remained viable longer than those at constant relative humidity of 55 per cent and deteriorated more rapidly than those at a constant relative humidity of 35 per cent. Seeds under conditions of alternation of humidity, then, were intermediate in germination capacity between those kept at the two humidities concerned. This same response applied to seeds alternated between 35 and 76 per cent relative humidity and between 55 and 76 per cent relative humidity. However, in these last two alternations when they were made 8-weekly, the seeds deteriorated as rapidly as they did when kept constant at the higher humidity. This was noteworthy in view of the fact that the seeds had been at either 35 or 55 per cent humidity, both of which permit retention of viability for fairly long periods (see Table II), for half of the time and con-

TABLE II

VIABILITY OF ONION SEEDS IN STORAGE AT 20° C. UNDER CONDITIONS OF CONSTANT AND ALTERNATING RELATIVE HUMIDITIES. GERMINATION AT BEGINNING OF THIS STORAGE TEST 96 PER CENT. FIGURES REPRESENT AVERAGES FROM DUPLICATES OF 100 SEEDS EACH USED FOR EACH TEST

Fluctuation		Per cent germination after total weeks of storage										
Period	Relative humidity	4	8	12	16	20	24	28	32	36	40	52
None	35	95	94	94	89	82	88	84	83	79	79	81
	55	98	86	93	87	45	28	19	10	11	7	—
	76	95	67	23	10	1	0	—	—	—	—	—
2-Weekly	35 to 55	96	92	92	86	72	84	69	65	57	42	34
	35 to 76	92	83	73	67	38	36	15	11	3	—	—
	55 to 76	93	77	71	45	6	3	1	—	—	—	—
4-Weekly	35 to 55	—	90	95	94	78	92	73	71	67	43	39
	35 to 76	—	86	90	57	58	35	18	9	10	2	—
	55 to 76	—	86	84	63	15	8	3	2	—	—	—
8-Weekly	35 to 55	—	—	—	90	—	86	—	75	—	61	—
	35 to 76	—	—	—	29	—	18	—	3	—	2	—
	55 to 76	—	—	—	14	—	4	—	0	—	—	—

sequently just half as long at the very harmful 76 per cent relative humidity as the control lot at this humidity.

Further examination of the deterioration effects after certain storage periods revealed that still other factors were involved. This is shown more clearly in Figure 2 in which the data are plotted in the form of graphs. Here

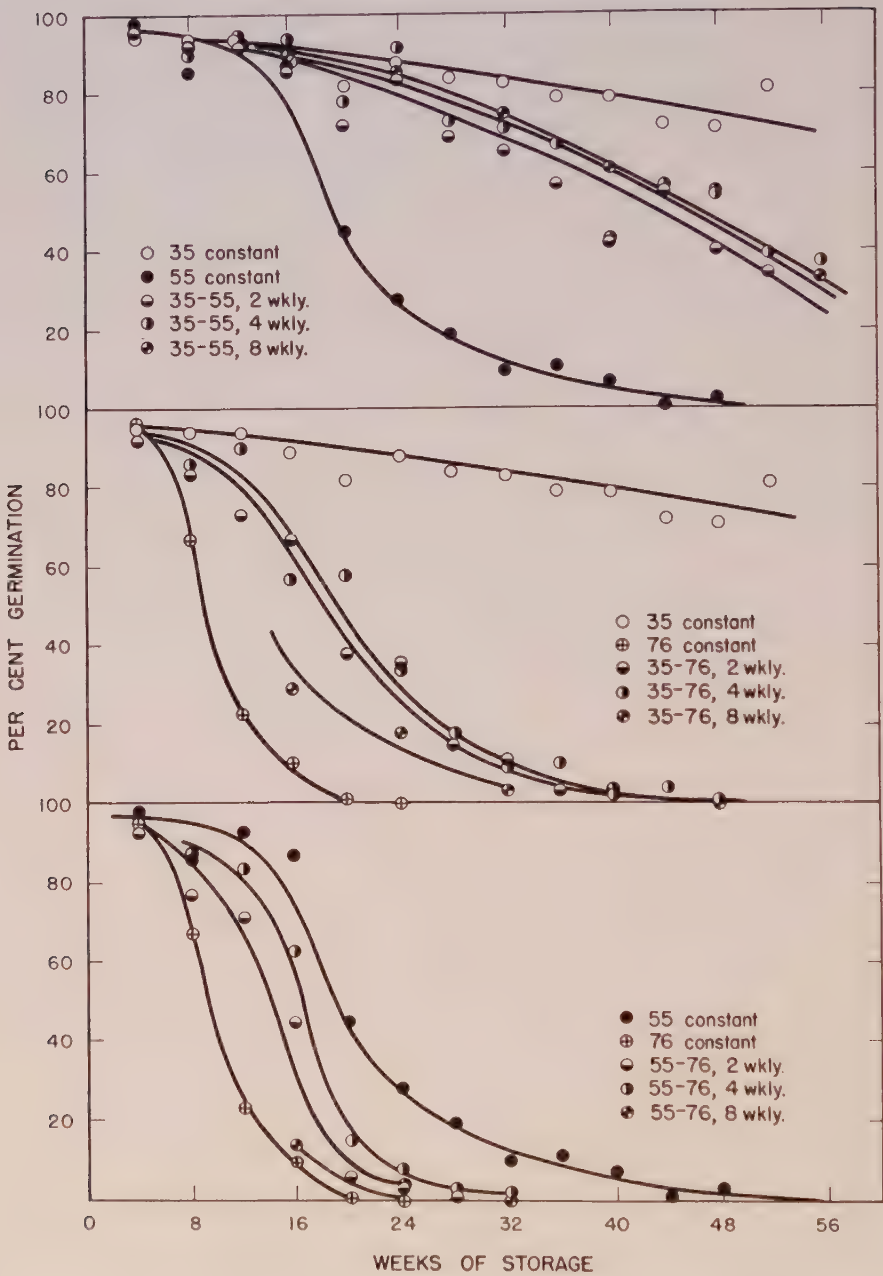

FIGURE 2. Viability of onion seeds stored under different humidity conditions.

it becomes evident that during the first 12 weeks of storage at 2- or 4-weekly alternations of either 35 to 76 or 55 to 76 the lower humidities acted favorably to prevent rapid loss of viability during the periods at 76 per cent. However, at the 16-week storage period and thereafter the lower humidities could no longer counteract the deleterious effect of the high humidity and the germination capacity curves more nearly approached that of the constant high humidity. In other words at 16 weeks of storage and thereafter deterioration at alternating humidities was at a more rapid rate than that expected from the behavior at each of the humidities. This was especially marked for the alternation of 35 to 76.

TABLE III

VIABILITY OF ONION SEEDS IN STORAGE AT 20° C. UNDER CONDITIONS OF CONSTANT AND ALTERNATING RELATIVE HUMIDITIES. FIGURES REPRESENT AVERAGES FROM DUPLICATES OF 100 SEEDS EACH USED FOR EACH TEST

Relative humidity		Per cent germination after weeks at high humidity							
%	Weekly alternation	4	8	12	16	20	24	28	32
35	None	95	94	94	89	82	88	84	83
55	None	98	86	93	87	45	28	19	10
76	None	95	67	23	10	1	0	—	—
35 to 55	2	92	86	84	65	42	40	—	—
	4	90	94	92	71	43	54	37	—
	8	—	90	—	75	—	55	—	10
35 to 76	2	83	67	36	11	—	—	—	—
	4	86	57	35	9	2	1	—	—
	8	—	29	—	3	—	0	—	—
55 to 76	2	77	45	3	—	—	—	—	—
	4	86	63	8	2	—	—	—	—
	8	—	14	—	0	—	—	—	—

On the other hand, at an alternation of 35 to 55 the periods at the favorable relative humidity of 35 per cent were sufficient to decrease the deterioration rate from that expected from the behavior at 55 per cent up to 48 weeks of storage after which the same effect as mentioned above was indicated by tests made after 52, 56, and 64 weeks of storage. This delay in the harmful effect produced by alternation of moisture content was no doubt due to the fact that 55 per cent relative humidity was much less harmful to germination capacity than 76 per cent.

The 8-weekly alternations deserve special consideration. The first viability test of these seeds was made after 16 weeks of storage. Since all seeds in alternating humidities were placed in the lower humidity first, this means that seeds at 55 to 76 alternation, for example, had the first 8 weeks at 55 per cent which permits high retention of viability and the second 8 weeks at 76 per cent. In spite of the fact that these seeds had only 8 weeks of the unfavorable 76 per cent after which, if no other factors were

involved, they should still have given 67 per cent germination, they gave only 14 per cent germination. A similar effect was obtained at 8-weekly alternation of 35 to 76 where the germination obtained was 29 per cent.

When the total length of time at the higher humidity, whether that time was obtained by alternation or by constant humidities, is considered, the germination percentages parallel the length of time except for the 8-weekly alternation which gives a much lower germination percentage as described above. These data are shown in Table III. The total period at high humidity, then, is of great importance in determining the length of life of onion seeds, but intervening periods of lower and hence more favorable humidities do not prolong the life span as much as would be expected from the behavior data of seeds kept constantly at these lower humidities.

If the total period at 76 per cent relative humidity in alternation of humidity were the only factor involved, 2-weekly, 4-weekly, and 8-weekly alternations should bring about the same deterioration when the total time at 76 is the same. Also an alternation of 35 to 76 per cent relative humidity should be as deleterious as 55 to 76. Such was not found to be the case. Therefore, some other factor must have been operating, and it appears from the data in hand that that factor was fluctuation in moisture content.

1942 TESTS

In the 1941 tests the moisture fluctuation in the seeds was from high to low at relatively short regular intervals, while in the case of the deterioration of seeds in storage containers opened at intervals for testing (1) the moisture content gradually changed from low to high or high to low depending upon the initial moisture content. Also in the latter case the change was over a much longer period. Another controlled experiment designed to resemble more closely the effect of opening sealed containers of seeds for sampling was set up in 1942. The seeds were kept in desiccators as described for the 1941 tests, but at 25° C. instead of 20° C. They were placed at either 35 or 55 per cent relative humidity at the beginning of the special storage period and were then transferred to different humidities at intervals of four weeks. The time at 35, 55, or 76 per cent relative humidity is shown in Table IV. The uncontrolled humidity was open storage in the laboratory. It will be seen that all of the seeds received a total of either 4, Nos. 5 to 24 inclusive, or 12 weeks, Nos. 25 to 42 inclusive, at 76 per cent relative humidity which has been demonstrated as unfavorable for the retention of viability. Onion, dandelion, and tomato seeds were used for these tests.

As in the 1941 tests tomato seeds remained fully viable except at a constant relative humidity of 76 per cent throughout the experiment. These data are not presented. Again dandelion seeds behaved in a manner similar to onion seeds, so that the discussion of these results will be con-

TABLE IV

VIABILITY OF ONION SEEDS IN STORAGE AT 25° C. UNDER VARIOUS HUMIDITY CONDITIONS. FIGURES REPRESENT AVERAGES FROM DUPLICATES OF 100 SEEDS EACH USED FOR EACH TEST

No.	Storage	Per cent germination after storage for weeks								
	Weeks at various humidities*	16	20	24	28	32	36	40	44	48
1	L48	90	90	92	87	84	81	85	87	81
2	M48	91	81	93	82	75	75	55	67	59
3	H48	72	47	15	—	—	—	—	—	—
4	Uncontrolled	94	79	86	81	64	75	56	72	75
5	L4 M4 H4 M4 L32	90	88	79	70	85	73	64	82	63
6	L4 M4 H4 M12 L24	—	—	86	81	79	71	59	79	67
7	L4 M4 H4 L36	85	94	77	65	81	69	68	78	75
8	L4 M12 H4 M4 L24	87	65	61	64	68	72	—	—	—
9	L4 M12 H4 M12 L16	—	—	—	—	73	57	58	70	68
10	L4 M12 H4 L28	—	80	86	72	80	75	56	—	—
11	L12 M4 H4 M4 L24	88	83	80	72	82	75	65	—	—
12	L12 M4 H4 M12 L16	—	—	—	—	77	75	64	60	69
13	L12 M4 H4 L28	—	96	88	77	66	67	77	—	—
14	L12 M12 H4 M4 L16	—	—	87	70	78	75	78	—	—
15	L12 M12 H4 M12 L8	—	—	—	—	—	—	63	76	69
16	L12 M12 H4 L20	—	—	—	89	79	74	70	—	—
17	M4 L4 M4 H4 M32	79	92	75	75	78	60	65	68	68
18	M4 L4 M12 H4 M24	—	94	79	68	68	68	69	55	49
19	M4 L12 M4 H4 M24	87	94	81	68	68	60	62	52	69
20	M4 L12 M12 H4 M16	—	—	—	71	69	56	68	55	57
21	M12 L4 M4 H4 M24	91	93	61	54	69	66	61	52	59
22	M12 L4 M12 H4 M16	—	—	—	80	81	65	62	59	54
23	M12 L12 M4 H4 M16	—	—	73	—	70	62	70	60	59
24	M12 L12 M12 H4 M8	—	—	—	—	—	77	72	61	54
25	L4 M4 H12 M4 L24	—	85	48	62	62	58	49	49	49
26	L4 M4 H12 M12 L16	—	—	—	—	57	53	37	42	45
27	L4 M4 H12 L28	—	76	60	41	61	49	—	—	—
28	L4 M12 H12 M4 L16	—	—	—	35	47	50	32	33	34
29	L4 M12 H12 M12 L8	—	—	—	—	—	—	20	29	20
30	L4 M12 H12 L20	—	—	—	40	45	34	25	26	—
31	L12 M4 H12 M4 L16	—	—	—	51	48	44	34	31	33
32	L12 M4 H12 M12 L8	—	—	—	—	—	—	39	34	31
33	L12 M4 H12 L20	—	—	—	55	48	45	39	27	—
34	L12 M12 H12 M4 L8	—	—	—	—	—	47	38	28	25
35	L12 M12 H12 L12	—	—	—	—	—	47	41	32	45
36	M4 L4 M4 H12 M24	—	—	41	44	43	33	39	26	22
37	M4 L4 M12 H12 M16	—	—	—	—	44	36	27	21	19
38	M4 L12 M4 H12 M16	—	—	—	—	47	44	31	22	21
39	M4 L12 M12 H12 M8	—	—	—	—	—	—	36	26	20
40	M12 L4 M4 H12 M16	—	—	—	—	42	38	27	21	19
41	M12 L4 M12 H12 M8	—	—	—	—	—	—	27	22	10
42	M12 L12 M4 H12 M8	—	—	—	—	—	—	31	15	21

* Per cent relative humidity, L = 35; M = 55; H = 76.

fined to onion only. Moisture determinations were made to ascertain the level attained in each humidity chamber. For onion this was found to be approximately 8 per cent at 35 per cent relative humidity, 10 per cent at 55 per cent relative humidity, and 15 per cent at 76 per cent relative humidity. Control lots were kept at constant relative humidities of 35,

55, and 76 per cent and open in the laboratory throughout the experiment. Storage, except the laboratory lot, was in a constant temperature chamber of 25° C.

The viability of onion seeds in storage under the various moisture conditions is shown in Table IV. The most obvious effect on viability as in the 1941 tests was the length of time at the very harmful relative humidity of 76 per cent. This was evident regardless of the condition of storage before or after exposure to this humidity. This means, then, that when the total storage time was considered, deterioration was more rapid at a con-

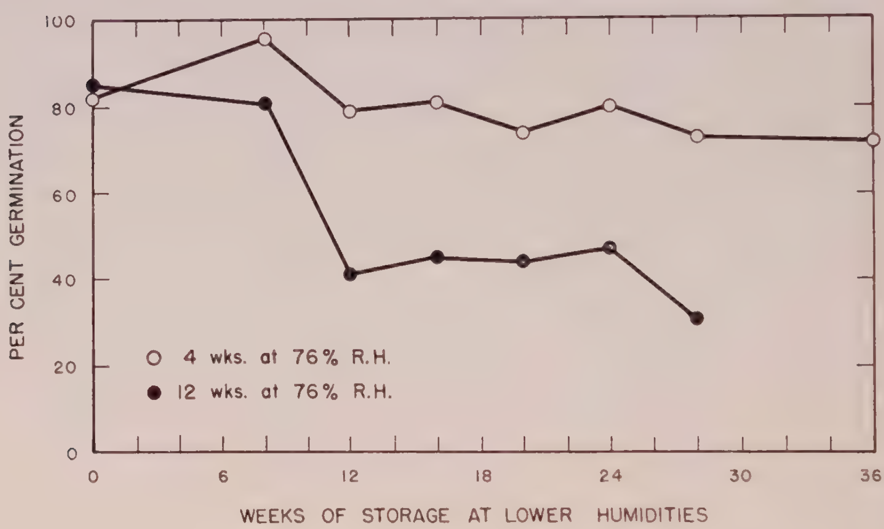

FIGURE 3. The effect of length of fluctuating storage humidities before exposure to 76 per cent relative humidity on subsequent viability of onion seeds at 76 per cent relative humidity. Average germination percentages obtained immediately after the period at 76 per cent relative humidity.

stant relative humidity of 76 per cent. However, when the total time at the high humidity alone was considered, germinative capacity decreased more rapidly when the seeds were subjected to moisture fluctuation. This is evident for seeds exposed for 12 weeks to 76 per cent relative humidity. But fluctuation in humidity prior to transfer accelerated deterioration at 76 per cent relative humidity only if it had been given for as long as 12 weeks before exposure to 76 per cent. Shorter periods of fluctuation before exposure were without effect as were the longer fluctuation periods when followed by only 4 weeks at 76 per cent relative humidity. This is shown graphically in Fig. 3.

Seeds stored open in the laboratory No. 4, Table IV, were also subjected

to a wide variation in moisture content. There was a steady increase in moisture from 6.7 per cent in May up to 11.1 per cent in August after which it declined to 9.3 per cent by the end of September and by November the moisture content was again 6.8 per cent, where it remained fairly constant for the remainder of the tests. Since this experiment was started in April, tests on germination were made at intervals of 4 weeks throughout the period of greatest fluctuation. The germination response was almost identical with that for storage at a constant humidity of 55 per cent, in spite of the fact that in the latter case seeds contained 11 per cent moisture throughout the storage period. In open laboratory storage this high amount of moisture was present for only approximately 4 weeks of the storage period.

In conclusion, then, it may be said that fluctuation in moisture content of stored onion seeds when continued for periods as long as twelve weeks and especially if a relatively high moisture content is involved is deleterious to their keeping quality and results in deterioration at a rate greater than that which would be expected from seed behavior at constant humidity levels.

SUMMARY

Seeds were stored under conditions of controlled temperatures and humidities to determine the effect of fluctation in moisture content on viability. The length of time at a harmful relative humidity (76 per cent) was found to be directly related to the deterioration rate of onion and dandelion seeds regardless of the storage conditions before and after this exposure. When the period of exposure to 76 per cent relative humidity was as long as eight or twelve weeks, previous variations in relative humidity accelerated loss of viability.

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THE STORAGE OF CITRUS SEEDS

Lela V. Barton

Although the longevity of many seeds in storage has been found to be increased by drying, there are some seeds which do not tolerate desiccation. A review of some literature pertaining to seeds of both of these types has been made by Crocker (4). Citrus seeds are among those which are injured by drying and hence deteriorate rapidly under ordinary conditions of storage. Dealers in these seeds have experienced difficulty in maintaining their germinative power although Florida sour orange seeds have been reported to be more resistant to drying effects than most other citrus seeds. For the most part they have kept the seeds moist, either in the original fruit juice or in moist sand or some other moist medium after cleaning until they were sold. Inquiries from seedsmen together with the fact that very little experimental work on the keeping quality of these seeds has been reported led to the present study. These experiments, designed to show the effects of moisture content, temperature, and sealing upon life span were somewhat limited due to the difficulty of obtaining large quantities of fresh seeds which had not been allowed to dry out. However, it has been possible to demonstrate that seeds of lemon and grapefruit may be kept viable for at least a year under certain conditions. Arrangements for supplies of seeds were made by Mr. Milo Hunt of Whittier, California.

1939 TESTS

Sweet orange and grapefruit seeds were obtained from Johnson-Appleby Co., Cambridge, Massachusetts, on April 30, 1939. They were received in slightly fermented juice from the fruits. The seeds had not been washed or dried after removal from the fruit. Many of them, especially orange seeds, had been injured in removal from the fruit. After they were received, the seeds were washed thoroughly and most of the broken ones removed. They were divided into two lots, both of which were dried on blotters in the laboratory, one for four hours and the other for forty-eight hours. At the end of each of these periods, moisture determinations and germination tests were made and lots were stored in open and in sealed containers in the laboratory and in a 5° C. room. Sealed storage was in tin cans with tight-fitting lids and sealing wax.

On April 24, 1939, seeds of rough lemon and sour orange were received from Charles T. McCarty, Eldred, Florida. These seeds had been cleaned and kept moist in shipment by mixing with moist sand. The sand was re-

TABLE I

GERMINATION OF CITRUS SEEDS IN MOIST GRANULATED PEAT MOSS AT 20° TO 30° C. FIGURES REPRESENT AVERAGES OF DUPLICATES FOR EACH TEST

Seed, date of storage	Storage: Moisture*	Storage: Temp.	Open storage 1939 May 11	Open 1939 May 24	Open 1939 June 27	Open 1939 July 25	Open 1939 Sept. 28	Open 1939 Dec. 21	Open 1940 Apr. 11	Open 1940 Sept. 12	Sealed storage 1939 May 11	Sealed 1939 May 24	Sealed 1939 June 27	Sealed 1939 July 25	Sealed 1939 Sept. 28	Sealed 1939 Dec. 21	Sealed 1940 Apr. 11	Sealed 1940 Sept. 12
Grapefruit May 3, 1939 32%**	123	Laboratory	—	3	2	—	—	—	—	—	—	18	0	—	—	—	—	—
		5° C.	—	32	54	51	44	36	16	0	—	35	46	35	14	0	0	—
Sweet orange May 3, 1939 71%**	82	Laboratory	—	26	3	—	—	—	—	—	—	39	0	—	—	—	—	—
		5° C.	—	64	62	31	15	15	0	0	—	69	63	39	1	0	0	—
Sour orange April 25, 1939 90%**	80	Laboratory	58	35	5	4	1	—	—	—	65	63	23	7	0	—	—	—
		5° C.	87	89	72	78	70	—	—	—	87	83	83	81	63	—	—	—
		−5° C.	27	16	1	0	—	—	—	—	28	12	1	0	—	—	—	—
Rough lemon April 25, 1939 84%**	56	Laboratory	35	36	36	34	16	15	4	1	71	69	12	7	0	—	—	—
		5° C.	80	85	67	56	59	21	14	0	89	72	69	84	67	79	62	53
		−5° C.	87	62	5	0	—	—	—	—	67	76	54	2	0	—	—	—

* Per cent moisture calculated on the basis of dry weight of seeds.
** Germination at time of storage.

moved by sieving and, after taking samples for moisture determinations and gemination tests, lots of each kind were stored immediately in open and sealed containers in the laboratory, in a 5° C. room, and in a room below freezing with an average temperature of −5° C. Other lots of these seeds were dried on blotters in the laboratory for seven days after which they were tested and stored like the moist samples.

For the moisture determinations made on all seeds at the time of storage, duplicate lots of approximately ten grams each were weighed and then dried in a vacuum oven at 78° C. for forty-eight hours, after which the dry weight was determined. Calculations of moisture content were made on the basis of the dry weights of the seeds. The moisture in the seeds stored in open containers gradually became adjusted to the humidity of the air in the storage room, but it was thought desirable to determine the effect of the initial drying on the keeping quality under all storage conditions. Seeds in sealed containers maintained the same moisture contents throughout the storage period since only one germination sample was sealed in a can and the seeds were never exposed to the air from the time they were stored until the germination test was made.

Germination tests after each storage period were made in the greenhouse and in controlled-temperature ovens at a daily alternating temperature of 20° to 30° C., where the seeds were left at 20° C. for sixteen hours and at 30° C. for eight hours daily. The medium used in the greenhouse was a mixture of sod soil, sand, and granulated peat moss in equal parts. For the oven tests, the seeds were mixed with moist granulated peat moss. Both of these methods proved satisfactory for testing the viability of stored seeds. Duplicates of fifty seeds each for each test were used for lemon, sweet orange, and sour orange seeds and duplicates of twenty-five seeds each were used for grapefruit.

More than one embryo was present in each of many of the seeds. In greenhouse plantings, the total number of seedlings appearing above ground were recorded. In oven cultures, only one germination per seed was counted and the seeds were discarded as soon as the first sprout appeared.

Results obtained from oven tests of moist seeds are shown in Table I. Each figure represents the average percentage from duplicates of seeds producing one seedling. Laboratory storage of moist grapefruit seeds resulted in rapid deterioration. On the other hand, seeds from the same lot which contained 123 per cent moisture at the beginning of the storage period remained fully viable in an open container at 5° C. for 232 days (December 21, 1939, Table I) after which the viability declined 50 per cent by 344 days (April 11, 1940). In sealed containers in this same room the seeds had deteriorated after 148 days (September 28, 1939) and gave no germination after 232 days. The moisture content of these moist seeds

in open containers was gradually reduced from 123 per cent to equilibrium with the air humidity in the room. This will be discussed further in 1942 tests below. In sealed containers the original high moisture level was retained, and was detrimental to keeping quality. Drying the seeds to 52 per cent moisture reduced the germination of grapefruit from 32 to 21 per cent. Such dried seeds were all dead in room temperature storage, open or sealed, when the first germination test was made 55 days after storage. From such seeds in open containers at 5° C. reduced germination was obtained after 83 days of storage and none were viable after 148 days. In spite of the initial decline in viability due to drying to 52 per cent moisture these seeds retained full germination capacity for 232 days in sealed containers at 5° C. This favorable effect of sealing was in direct contrast to the effect on seeds with 123 per cent moisture.

Sweet orange seeds with 82 per cent moisture behaved in a manner similar to the moist grapefruit already described. Again open storage in a 5° C. room was effective in preserving the seeds for a longer time than sealed storage while open or sealed storage in the laboratory was very unfavorable (Table I). Reduction in moisture of orange seeds from 82 to 25 per cent of their dry weight decreased their germination power from 71 to 9 per cent none of which survived 53 days' storage either in the laboratory or at 5° C.

Sour orange seeds containing 80 per cent moisture and capable of 90 per cent germination exhibited greater resistance to degeneration in storage in the laboratory and at 5° C. than either grapefruit or sweet orange (Table I). The supply of sour orange seeds stored at 5° C. was exhausted before any differences in open and sealed storage were apparent. After 156 (September 28, 1939) days of storage when the last germination test was made seeds from these two conditions showed 70 and 63 per cent viability. In addition to the laboratory and 5° C. storage, −5° C. was also used for sour orange seeds. This temperature proved even more harmful than that of the laboratory, causing a decline in viability from 90 to 27 per cent in open storage in 16 days. Sealed storage below freezing produced similar effects.

Not only were sour orange seeds more tolerant of laboratory temperature storage than either grapefruit or sweet orange, but they also withstood drying to 4 per cent moisture, after which they were still capable of 22 per cent germination. After such drying these seeds could not endure storage in open containers for as long as 57 days at any of the three temperatures tried, but sealed storage at 5° C. kept them viable for 346 days in spite of their reduced germination power at the beginning of the storage period.

The response of moist lemon seeds (56 per cent moisture) to storage was similar to that of the sour orange. More lemon seeds were available for tests and demonstrated that sealed storage was superior to open stor-

age at 5° C. for keeping moist seeds viable for a period as long as 352 days (April 11, 1940, Table I). It should be pointed out, however, that "moist" lemon seeds contain 56 per cent moisture as compared to 80 per cent in the sour orange and even higher percentages in the sweet orange and grapefruit. Although open storage of moist lemon seeds in the laboratory caused almost immediate reduction in viability, it was superior to sealed storage at this temperature as far as length of life was concerned.

Lemon seeds were even more resistant to drying than sour orange seeds. The former were dried from 56 per cent moisture to 4 per cent with a resulting decrease in germination from 84 to 46 per cent. In sealed storage in the laboratory and at 5° C. these seeds retained their full germination capacity for 500 days at which time the seed supply was exhausted. About one-third of the seeds failed to sprout after the same length of time at −5° C. Deterioration of dried seeds was much more rapid in open storage at all temperatures.

1942 TESTS

Twenty-five pounds of grapefruit seeds still moist with the juice of the fruit and with some of the fruit pulp still attached were received from Johnson-Appleby Co. on February 16, 1942. The seeds were in excellent condition. No fermentation had taken place. They were rubbed and washed thoroughly to free them of pulp the same day they were received. They were then left moist overnight at 10° C. The next day storage tests were begun. Some seeds were stored moist containing 131 per cent moisture calculated on the basis of the dry weight of the seeds. Others were dried on blotters in the laboratory for six, sixteen, and twenty-four hours before storage. At the end of the drying periods the moisture remaining in the seeds was 100, 78, and 60 per cent respectively. Further drying was avoided because the seed supply was limited and the 1939 tests had shown that reduction in moisture to 52 per cent resulted in reduced germination of grapefruit.

The four lots were divided into individual viability test samples and were stored in open and sealed containers in the laboratory, at 5° C., and at approximately −5° C. Moisture determinations and germination tests as described for the 1939 experiments were made at the beginning of storage. Further germination tests were made after storage for 2, 4, 8, 12, 16, and 52 weeks. For the most part, duplicates of thirty-five seeds each were used for viability tests both in moist granulated peat moss at a daily alternating temperature of 20° to 30° C. and in soil in the greenhouse. Seedling counts were made as described for 1939.

Results from oven tests are shown in Table II. It is evident that none of the drying periods was injurious to the seeds since 94 to 99 per cent of them germinated when tested immediately after reduction in moisture content.

TABLE II

GERMINATION OF GRAPEFRUIT SEEDS IN MOIST GRANULATED PEAT MOSS AT 20° TO 30° C. FIGURES REPRESENT AVERAGES OF DUPLICATES FOR EACH TEST

Storage			Per cent germination after storage for weeks						
Temperature	Moisture*	Open or sealed	0	2	4	8	12	16	52
Laboratory	131	O	94	68	94	13	13	32	0
		S		94	89	16	4	0	—
	100	O	96	80	66	13	4	24	—
		S		89	77	9	0	—	—
	78	O	99	84	86	24	43	54	3
		S		99	97	80	50	9	—
	60	O	94	93	83	10	36	36	1
		S		90	93	82	90	98	0
5° C.	131	O	94	100	97	93	87	89	89
		S		99	97	87	77	86	0
	100	O	96	97	100	94	94	83	88
		S		100	97	97	84	84	41
	78	O	99	97	100	92	97	91	83
		S		100	99	99	79	91	73
	60	O	94	94	97	93	90	89	88
		S		88	88	91	94	84	66
−5° C.	131	O	94	76	84	71	3	0	—
		S		85	79	83	3	0	—
	100	O	96	76	81	87	3	0	—
		S		77	77	74	3	0	—
	78	O	99	87	94	86	0	1	—
		S		76	89	74	1	0	—
	60	O	94	93	89	86	0	0	—
		S		91	99	89	0	0	—

* Per cent moisture calculated on the basis of dry weight of seeds.

First let us consider the life span in the laboratory. Open and sealed storage had similar effects on the keeping quality of the moist seeds and those dried to 100 per cent moisture. The germination power was reduced significantly under these conditions after eight weeks. These seeds were so very moist that some of them germinated in the open storage containers before they dried out. No germinations occurred in sealed storage, however, regardless of moisture content, due, probably, to the lack of oxygen or the accumulation of carbon dioxide in the storage chamber. When the seeds were dried to 78 per cent moisture before storing, their life span at room temperature was lengthened. Further reduction in moisture content to 60 per cent before storage made it possible for 98 per cent of the seeds

to germinate after sixteen weeks in sealed containers. On the other hand, open storage of these seeds in the laboratory brought about a marked reduction in germination after eight weeks. For effective storage of grapefruit seeds for as long as sixteen weeks in the laboratory, it was necessary to reduce the moisture to about 60 per cent and then place in sealed containers. Sealing tended to be harmful rather than beneficial to seeds with higher moisture contents. The limit of effective drying previous to storage was not determined by these experiments. However, the 1939 tests reported above indicated that drying to 52 per cent moisture injured grapefruit seeds. Since the crops may vary in their resistance to desiccation, more work needs to be done to settle this point.

A temperature of 5° C. was superior to laboratory temperature for prolonging the life of grapefruit seeds (Table II). Again, as in the 1939 tests, open storage at this temperature proved an excellent means for keeping the seeds viable for at least a year, at which time the seed supply was exhausted. The initial storage moisture present in the seeds was without effect on longevity under these conditions. This was to be expected since the seeds had not been harmed by the drying process and since they were in open containers. In sealed containers, however, the advantage of pre-desiccation to at least 78 per cent moisture was demonstrated. Germination tests of samples from sealed containers at 5° C. of seeds with moisture contents of 131, 100, 78, and 60 per cent showed 0, 41, 73, and 66 per cent viable at the end of a year of storage. Such a difference was not evident after sixteen weeks of storage at which time all of the seeds gave excellent germination.

Grapefruit seeds were injured by a temperature below freezing so that only occasional seedlings were produced after twelve weeks of such storage (Table II). No differences were observed between open and sealed storage at this temperature. These effects were in agreement with those obtained for sour orange and rough lemon seeds described above.

DISCUSSION

Other workers have dealt with short-lived seeds and have attributed their deterioration to various causes.

Jones (5) reported that the viability of river maple (*Acer saccharinum* L.) seeds was lost if their moisture content was reduced to 30 to 34 per cent. Since this occurred at temperatures from 0° to 35° C., he concluded that the temperature was not important but that life depended entirely on water content. Seeds harvested in the spring and stored over water in desiccators at 10° C. continued to give high germination up until November. They kept better when carbon dioxide was not allowed to accumulate.

On the other hand, Kidd (6) attributed prolongation of the life of

rapidly-deteriorating *Hevea brasiliensis* seeds to the presence of 40 per cent of carbon dioxide produced by the respiration of the seeds in a closed flask. He did not make moisture determinations of these seeds but said that they had a high water content. He summarized, in part, that "The resting stage of the moist seed is primarily a phase of narcosis induced by the action of carbon dioxide" (6, p. 624).

Busse (3) thought that the rapid loss of viability of poplar seeds when left in air was due to the injurious action of oxygen. He was able to demonstrate that seeds in sealed containers with reduced oxygen pressure remained viable longer than those subjected to full atmospheric pressure.

Work with grapefruit seeds in this laboratory has shown the importance of moisture content, temperature, and sealing effects on viability. High moistures were detrimental to seeds in closed containers especially when stored at laboratory temperatures. However, drying of grapefruit seeds to 60 per cent moisture, still a very high moisture content, prolonged their germination capacity in sealed storage in the laboratory as compared with open storage at this temperature. Obviously, then, some factor other than carbon dioxide accumulation was operating to prolong the life of grapefruit seeds, since sealed storage was not favorable in all cases. This was shown further by the less favorable effect of sealed storage at 5° C. as compared with open storage at that temperature. Mold appeared on moist seeds in sealed storage in the laboratory at an early date and may have been a factor in deterioration but it would be very difficult to say whether the mold led to deterioration or whether deterioration led to the development of mold.

The best storage condition found for grapefruit seeds was in open containers in a room held at 5° C. This room had a very humid atmosphere. After a year of open storage grapefruit seeds contained 18 per cent moisture. Since limited tests indicated that these seeds were somewhat injured by drying on blotters in the laboratory to 52 per cent moisture, the beneficial effect of drying to 18 per cent at 5° C. is of particular interest. Certainly it cannot be said that their longevity depends entirely upon water content. It appears that the drying temperature and perhaps other factors such as rapidity of desiccation etc. are of importance. In this respect, these seeds differ from those of river maple as described by Jones (5). Citrus seeds apparently vary widely in their tolerance to desiccation. Whereas the germination of seeds of grapefruit was reduced 30 per cent by drying to 52 per cent moisture and those of sweet orange 80 per cent by drying to 25 per cent moisture, sour orange and rough lemon seeds could be dried to 4 per cent and still retain 25 and 50 per cent of their original germination power.

The harmful effect of a temperature of approximately −5° C. on the keeping quality of seeds of grapefruit, sour orange, and rough lemon is in

direct contrast to the efficacy of this same temperature for maintaining the viability of many seeds of relatively low moisture contents, such as vegetable and elm seeds for example (Barton 1, 2). Other authors have also reported favorable storage effects of temperatures below freezing (Crocker 4). That the deleterious effect on citrus seeds of such temperatures was not due entirely to high moisture content was demonstrated in the present experiment where rough lemon and sour orange seeds dried to 4 per cent moisture and still capable of germination were unfavorably affected.

The interrelation of gaseous exchange, moisture content, and temperature in their effects on the keeping quality of short-lived citrus seeds is not apparent at the present time. Much more work is needed to clarify the situation.

SUMMARY

Seeds of grapefruit, sweet orange, sour orange, and rough lemon were stored under different conditions to determine factors which would prolong life. It was found that grapefruit and sweet orange seeds were injured by drying on blotters in the laboratory to 52 and 25 per cent moisture respectively calculated on the basis of the dry weight of the seeds. However, seeds of both of these forms remained viable longest in open storage in a humid atmosphere at 5° C. where the moisture content was reduced to approximately 17 per cent. Grapefruit seeds may be kept successfully for at least a year under these conditions. Five degrees C. also proved best for maintaining the viability of sour orange and rough lemon seeds both of which tolerated more drying than grapefruit and sweet orange seeds. Laboratory temperature and $-5°$ C. were deleterious to keeping quality. Some phases of the interrelation of gaseous exchange, moisture content, and temperature in their effects on the longevity of short-lived citrus seeds are discussed.

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INACTIVATION OF THE BROWNING SYSTEM IN DRIED APPLES

F. E. Denny

In a previous article (2) it was shown that the browning, which occurs when the cut tissue of apple (*Pyrus malus* L.) is exposed to air, could be prevented by dipping the freshly-cut fruit tissue into a solution made by dissolving one gram of thiourea, NH_2CSNH_2 (also called thiocarbamide), in a liter of water. It was noted at that time, however, and has since been demonstrated many times with apple tissue dried after the thiourea treatment, that the browning system, as a whole, of such dried products was not inactivated, at least not completely, since if such dried apples were soaked in an excess of water the tissue promptly developed a brown color.

The object of the present paper is to show that if the dried apple slices, obtained as a result of the thiourea treatment, are heated for approximately 1.5 hours at 80° C. (176° F.), the tissue may then be soaked in water without the development of a brown color. The function of the thiourea is to protect the cut surface from browning during drying, the inactivation of an essential component of the browning system of the internal portions of the tissue being then accomplished by means of heat.

It is also shown that by the use of thiourea a dried apple pulp in the form of thin sheets may be obtained, and that when this dried product is soaked in water there is no development of a brown color in the pulp. A heat treatment of the dried product is unnecessary when the proper amount of thiourea is incorporated into the pulp at the time the fresh tissue is disintegrated in preparation for the drying process.

EXPERIMENTS WITH SLICES OF APPLES

Preparation of dried slices. The apples were peeled and the cores removed. Slices uniformly one-quarter inch thick were prepared by the use of a hand-operated stainless steel slicing machine. These were dipped at once into a beaker partly filled with a solution made by dissolving one gram of thiourea in a liter of water. The slices from one apple were allowed to stand in the dipping liquid while the slices from the next apple were being prepared (approximately 30 to 60 seconds). These apple rings were then arranged on a half-inch wooden rod at distances of about one-quarter inch apart and were placed on a rack in the drying room. The temperature of the room was thermostatically controlled at 35° C. (95° F.) and the air

was stirred briskly but not violently by the heat-control equipment. Under these conditions the moisture content of the apple slices was reduced until it was about 15 per cent, in about 24 hours. Usually samples were removed at different intervals to furnish tissue with varying amounts of moisture. It was found necessary to reduce the moisture content to below 20 per cent in order that good color of slices could be maintained on further standing in air at room temperature.

The apple slices prepared and dried in this way were white, without any tinge of brown color. This is shown by the dry samples in tier 2, Figure 1 A. However, although these slices were white (and have been found to remain so for many weeks if kept dry, and indefinitely if stored dry at a temperature of 5° C.), the browning system in the bulk of the tissue had not been inactivated by the treatment. When the dry white slices were placed in water and soaked for a few hours the tissue became brown, as shown in tier 3, Figure 1 A. The thiourea treatment had protected the outer layer of the slice until the moisture content had been reduced to the point at which browning in the internal region could not occur.

Heating of dried slices. The inactivation of the browning system in these dried apple slices was accomplished by heating them in an oven. For these tests only the central slices from each apple were used. Browning is particularly active in the tissue adjacent to the core and in the fibrovascular strands in the center of the apple. Four or five of these central slices were available from each apple.

Half-pint glass fruit jars were used as containers for heating dried apple slices in the oven. The empty jars were first placed in the electric oven and were brought to the desired temperature as shown by a thermometer placed approximately one inch from the edges of four such jars. Randomly selected apple rings were put in the jars which were sealed and returned quickly to the oven. After the desired period of heating, the slices were removed from the oven, placed in an excess of water in beakers, and soaked. During the first two hours of soaking the water was decanted occasionally and fresh water was applied. This was to leach the thiourea from the tissue. The soaking was continued overnight and the color of the apple slice was then noted.

Preliminary tests indicated the approximate ranges of time, temperature, and moisture content of slices for successful inactivation of the browning system. At 85° C. (185° F.), if the moisture content was above 15 per cent, caramelization occurred when the duration of heating was as much as two hours. At 75° C., two hours of heating were insufficient to prevent browning of soaked tissue if the moisture content of the dried slices at the time of heating was below 15 per cent. A heating period of one to two hours at 80° C. (176° F.) was found to be about right. The results of such a test with tissue from five varieties of apples are shown in tiers 4 to 7,

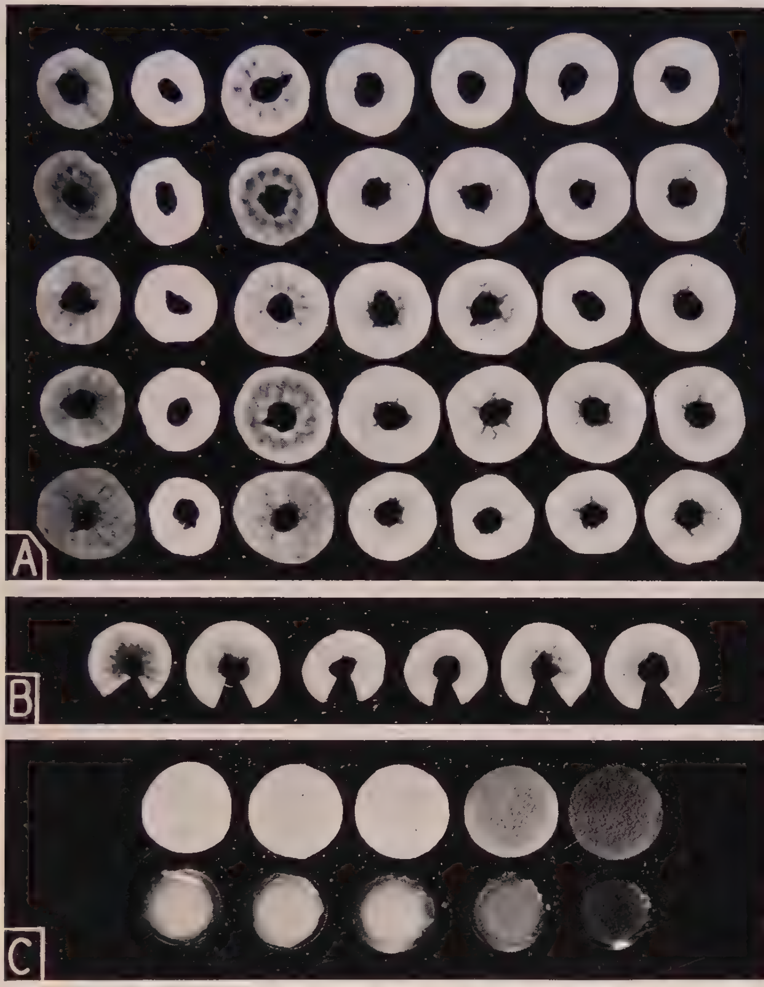

FIGURE 1. A. Rows: top row var. McIntosh and in order downward: Northern Spy, Cortlandt, King, Rhode Island Greening. Tiers: No. 1, control slices, not treated, dried, soaked in water; Nos. 2 to 7, slices dipped into 0.1 per cent thiourea solution and dried; No. 2, not soaked; Nos. 3 to 7, soaked in water; No. 3, not heated before soaking; Nos. 4 and 5, heated 1 hour at 80° C. after drying and before soaking; Nos. 6 and 7, heated 2 hours; Nos. 4 and 6, slices with 14 per cent moisture, Nos. 5 and 7 with 11 per cent moisture at time of heating. B. Ring No. 1 at left dipped into 0.1 per cent thiourea, dried, not heated, soaked in H_2O; rings Nos. 2 to 5 dipped into 0.1 per cent thiourea, dried and heated at 80° C. for 1.5 hours before soaking in water; No. 6, same but heated 2 hours. Moisture contents of rings before heating were, left to right: 17.5, 13.5, 9.9, 8.0, 8.0. C. Films of dried apple pulp: top row, dried, not soaked in water; bottom row, corresponding lots after soaking in water. Amount of thiourea added to fresh tissue at time of pulping: left to right, 1 part by weight of thiourea to 5000 parts of fresh tissue; 1:7500; 1:10,000; 1:12,500; control, water added instead of thiourea solution.

Figure 1 A. The heating period after drying and before soaking was one hour for the lots shown in tiers 4 and 5, and two hours for those shown in tiers 6 and 7. Two different moisture contents were also involved in this test, the moisture content of the lots in tiers 4 and 6 being 14 per cent, and those in tiers 5 and 7 being 11 per cent. It is seen that two hours' heating at 80° C. (176° F.), tiers 6 and 7, was sufficient to inactivate the browning system of the tissue of all five varieties, and at both moisture contents, since slices so treated when soaked in water retained their light color in a satisfactory manner. The heating period of one hour was also successful in all cases but one, this being the Cortlandt sample which showed an undue amount of browning in the series with 11 per cent moisture, tier 5, row 3, Figure 1 A.

Selecting a heating period of 1.5 hours at 80° C. (176° F.), the range of moisture contents of tissue at which inactivation could be accomplished was tested. In this experiment, apples of the variety McIntosh were used. The thiourea dipping solution was again 0.1 per cent, and samples of apple rings were removed at intervals after the drying process was started so as to furnish lots with decreasing amounts of moisture; the actual moisture contents of these lots were found later to be: 17.5, 13.5, 9.9, and 8.0 per cent. The results of this test are shown in Figure 1 B. The missing segments in Figure 1 B represent those removed before soaking, for the purpose of a moisture determination. The four rings in the center show the condition after heating for 1.5 hours at 80° C. (176° F.) and soaking in water. Since the lot at 8.0 per cent moisture (second from the right in Fig. 1 B), showed a small amount of browning, another sample at this moisture content was heated for 2 hours instead of 1.5 hours with the result that complete inactivation of the browning system was obtained (right hand ring in Fig. 1 B).

As a result of these tests, the method of inactivating the browning system in dried apples may be stated as follows: slice the peeled and cored apples into one-quarter inch slices, dip into a thiourea solution made by dissolving one gram of thiourea in a liter of water, dry the slices in a current of air until the moisture content is 10 to 15 per cent, heat the dried slices in an oven at 80° C. (176° F.) for 1.5 hours.

Effect on constituents of the browning system. Although the main object in these tests was to obtain a dried apple product which would not turn brown when it was soaked in water, some tests were made to determine which part or parts of the browning system were inactivated by the heat treatment. The procedure used was that employed by Overholser and Cruess (3) in testing for the organic peroxide and peroxidase components of the oxidase system. According to this method a blue or purple color resulting from the addition of the benzidine reagent alone indicates the presence of both organic peroxide and an active peroxidase; but if the

coloration is not obtained until hydrogen peroxide is added in addition to benzidine, the test indicates the absence of a naturally-occurring organic peroxide but the presence of an active peroxidase.

Thiourea-treated dried apple rings white in color were soaked in an excess of water which was then decanted and a few drops of the benzidine reagent were applied. A dark blue coloration resulted within a few minutes, indicating the presence of both peroxidase and a naturally-occurring peroxide. When the same test was applied to the apple rings which had been heated at 80° C. for 1.5 hours the benzidine test before the addition of hydrogen peroxide was negative, but it was strongly positive after applying both benzidine and H_2O_2. These tests indicate that the heating process had destroyed the organic peroxide constituent but not the peroxidase constituent of the browning system. This shows that the functioning of the browning system, as a whole, could be prevented by inactivating only one of the components of the system, i.e., the organic peroxide. Browning could then not occur even though the peroxidase component remained active.

EXPERIMENTS WITH APPLE PULP

When slices of apple fruit are merely dipped into a dilute thiourea solution, it is only in the outer cells of the slices that thiourea comes into contact with the browning system. As shown in the preceding paragraphs, the internal tissues of the slices retain their capacity for browning when soaked in water.

This leads to the question whether subsequent browning could be prevented by a thorough disintegration of the tissue in the presence of thiourea previous to the exposure to drying conditions.

In the first experiments on incorporating thiourea into apple pulp, the thiourea solution was added to the chopped fresh tissue (10 cc. of solution to 100 g. of fruit), and after grinding with a mortar and pestle, the pulp was poured on a piece of cheesecloth laid on a large watch glass, and dried in a current of air at 35° C. (95° F.).

When the thiourea added amounted to at least 1 part of thiourea (by weight) to 10,000 parts of apple tissue, the film of dried pulp so obtained was of good color, i.e., browning did not occur. When several small quantities of this dried pulp were each separately soaked in water, however, some of these small samples showed some development of brown color, while others did not. This indicated that the grinding had been incomplete, that small bits of tissue survived the grinding in such manner that the thiourea did not penetrate to the inner portions. Such bits then released their content of protected enzyme when the tissue was soaked in water.

This difficulty was overcome by the use of a Waring Blendor, see Davis (1). The procedure employed was as follows: the apples were peeled and cored; 200 g. of tissue in the form of small chunks were placed in the bowl

of the machine and 60 cc. of the thiourea solution were added. The apparatus whipped the tissue into the condition of a fine pulp within a few minutes, and this pulp was poured on cheesecloths placed on eight-inch watch glasses. In a current of air at 35° C. (95° F.) the tissue dried in about 24 hours. The cloth was stripped from the watch glass, and in turn, the film of dried apple pulp was stripped from the cloth.

When the amount of thiourea was varied in series to furnish 1 part (by weight) of thiourea to 5000, 7500, 10,000 and 12,500 parts of fresh apple tissue, it was found that the limit of dilution to give complete inactivation of the browning system, i.e., no coloration when the dried tissue was soaked in water, was at 1 part to 10,000. At 1:12,500 considerable browning occurred.

Discs of these samples of dried apple pulp were cut out from these films and the colors before and after soaking in water are shown in Figure 1 C. The products obtained with thiourea treatments using amounts of 1:5000, 1:7500, and 1:10,000 (by weight) were of good color in the dried condition, and no browning occurred with them when they were soaked in water.

Effect on constituents of the browning system. The benzidine and benzidine $+H_2O_2$ tests were applied to these films of dried apple pulp in the manner previously described for the dried apple slices. Portions of the dried films from the 1:5000, 1:7500, and 1:10,000 lots were soaked in an excess of water which was then decanted. The application of a few drops of the benzidine reagent did not lead to the development of a blue color, indicating the destruction of the organic peroxide constituent; but upon the addition of both benzidine and H_2O_2 a dark blue color developed promptly, showing that an active peroxidase was present in the tissue.

Samples of dry films from these same lots of pulp were heated in an oven at 80° C. (176° F.) for 1.5 hours. A repetition of the benzidine tests gave negative responses both before and after the addition of hydrogen peroxide, indicating that during the heating of the dried pulps even the peroxidase constituent of the browning system had become inactivated.

Thus, in the effect of heat upon the dried tissue, there was a difference between the dried slices (in which thiourea came in contact with only the surface cells of the fresh slices) and the dried pulps (in which the thiourea came into contact with perhaps nearly all of the fresh tissue). The heat did not inactivate the peroxidase constituent of the slices but did inactivate it in the pulps. With dry slices, the organic peroxide constituent was not inactivated until after heating, with dry pulp it was inactivated even before heating. With slices the peroxidase component was still active even after heating, but with the pulp the heat treatment rendered it completely inactive.

SUMMARY

The white dried apple slices obtainable when fresh apple slices are dipped into a 0.1 per cent thiourea, NH_2CSNH_2 (thiocarbamide), solution and dried in a current of warm air, turn brown if they are soaked in water. However, when the thiourea-treated dried slices were heated in an oven at 80° C. (176° F.) for 1.5 hours, browning did not occur when the slices are soaked in water.

Benzidine tests (with and without hydrogen peroxide) showed that the organic peroxide and peroxidase constituents of the browning system were both present in the active condition in the dried slices previous to heating, and that the heat treatment destroyed only the peroxide component, the peroxidase constituent retaining its activity.

When thiourea was added to fresh apple tissue which was then rapidly reduced to a pulp in the bowl of a mixing machine with rotating blades, the pulp when dried in shallow layers in a current of warm air furnished a dried apple product in the form of films. These films of dried apple pulp may be soaked in water, the resulting sauce showing no brown color. The lowest amount of thiourea which will give this result is approximately 1 part of thiourea to 10,000 parts of fresh apple fruit tissue (by weight).

Benzidine tests on the dried pulp before heating showed that the organic peroxide component of the browning system had been destroyed by the thiourea treatment, although the peroxidase constituent was still functional. Heating the film of dried pulp at 80° C. for 1.5 hours resulted in the inactivation of the peroxidase.

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EFFECT OF POST-HARVEST PRE-STORAGE CONDITIONS ON THE RATE OF DEVELOPMENT OF SUGAR IN POTATO TUBERS DURING SUBSEQUENT COLD STORAGE

F. E. Denny and Norwood C. Thornton

Previous experiments (2, 3) showed that the time after harvest at which potato tubers (*Solanum tuberosum* L.) were placed in cold storage (at 5° C.) was an important factor in the subsequent rate of sugar development, early storage inducing the formation of high amounts of reducing sugar, while a late start of storage favored the accumulation of sucrose.

The present experiments were carried out to determine whether the conditions of storage from harvest time until cold storage was initiated had an influence on the changes in sugar content during cold storage.

Shortly after harvest the samples of tubers were placed in each of four rooms with temperatures ranging from 15° to 24.4° C. At the highest temperature the relative humidity was varied from 42 to 92 per cent. At intervals samples were transferred from these rooms to cold storage at 5° C., and sugar analyses of the juice made at intervals thereafter.

The principal effect of these pre-storage conditions was upon the sucrose content during subsequent cold storage. The lowest post-harvest temperature used, i.e., 15° C., retarded the rate of sucrose accumulation, the samples from this storage condition forming only about one-half as much sucrose as was formed by the tubers stored under the other three sets of conditions.

A surprisingly small influence of the post-harvest storage condition upon the reducing sugar content in subsequent cold storage was found. There was some evidence, however, that pre-storage at 15° C. held the reducing sugar content of the tubers to a level about 20 per cent lower than that of tubers from the other three pre-storage conditions.

It should be emphasized that the post-harvest pre-storage conditions tested did not influence the content of either reducing sugar or sucrose during the pre-storage period. The effects found were upon the sugar content after the tubers were transferred from the pre-storage conditions to cold storage at 5° C.

MATERIALS AND METHODS

Tubers. The tubers of the varieties Green Mountain and Carman No. 3 were grown in the Institute gardens and were harvested in mid-August

1942. Those of the varieties Irish Cobbler and Katahdin were purchased in a local market from supplies of tubers that had been recently harvested in Long Island.

The tubers were sorted into sizes and divided proportionately into samples of 16 tubers each for Irish Cobbler and Katahdin, 18 tubers for Green Mountain, and 20 tubers for Carman No. 3. The tuber samples in cheesecloth bags were distributed into burlap bags for storage under the conditions of the test.

Storage rooms. The rooms used for obtaining the variations in temperature and humidity were as follows: Room A was a warm room, the temperature averaging 23.3° C. in the interval from September 3 to December 24, and varying only about 2° from this value. The air was dry, the relative humidity being about 42 per cent, and varying from about 53 per cent in September to about 43 per cent in October, 39 per cent in November, and 23 per cent in December.

Room B was a galvanized iron cylinder, capacity 790 liters, placed in room A. The humidity of this space was kept high by suspending wet cloths from the ceiling. The average temperature was 24.4° C., slightly higher than that of room A. The humidity was 92 per cent, varying from about 91 per cent in September, to about 94 per cent in October, 93 per cent in November, and 87 per cent in December. Slow ventilation was provided and gas analyses were made frequently to make certain that there was no accumulation of carbon dioxide within the cylinder.

Room C was a basement room of the cellar type with earth completely surrounding it except on one wall. Constancy of temperature was not maintained but the changes were quite gradual, ranging from 22.5° C. in early September to 18.5° C. in late October, at which time electric heat was applied which maintained a temperature not lower than 17° C. at any time until the final sample was removed on December 24. The average temperature for the entire period was 19.8° C. The average relative humidity of this room was 70 per cent, varying from 85 per cent in September to 76 per cent in October, 63 per cent in November, and 43 per cent in December.

Room D was a well insulated thermostatically controlled storage room with the temperature maintained at 15° C., and deviating only slightly at any time from this figure. The average humidity was 86 per cent, varying from 88 per cent in September to 87 per cent in October, 84 per cent in November, and 80 per cent in December.

Durations in pre-storage. The tubers were placed under the pre-storage conditions on August 27. Therefore the lots removed on October 1 and placed in cold storage were under the pre-storage conditions for 35 days, those removed October 26 for 60 days, those removed December 1 for 96 days, and those removed on December 24 for 118 days.

Sugar analyses. After removal from the storage rooms the tubers were washed, dried, and minced in a food grinder. The juice, obtained by squeezing the tissue in cheesecloth, was centrifuged, and 25 cc. samples were taken for analysis. The analytical procedure used for the reducing sugar and sucrose was the same as that previously described (1, p. 294).

RESULTS

Reducing sugar. The gain in reducing sugar shown by samples removed at intervals from the various rooms is shown in Table I. Since the tubers which were removed from the rooms at the end of the pre-storage period were practically devoid of reducing sugar at that time, the values in Table I represent not only the gains during the intervals in cold storage, but also the actual reducing sugar values in milligrams per cc. of juice at the time of analysis. For example, 59 of the 64 samples removed from the four rooms A, B, C, and D on October 1, October 26, December 1, and December 24, showed less than 0.1 mg. of reducing sugar per cc. of juice, 63 out of 64 showed less than 0.4 mg., and only one showed a higher amount this one being the Green Mountain sample from room D at the December 24 starting date, the value in this case being 1.7 mg.

The values opposite the entries "Room total" in column 1 show the totals for each room for each of the three cold storage duration intervals, 15, 29, and 60 days. The analysis of variance for each of these three intervals is shown in Table III, columns 1 to 5. It is only at the 60-day interval that significant differences among the rooms are found. The gains in reducing sugar for room D (pre-storage at 15° C.) were less than those for the other rooms. This result is due mainly to the small increase made by the tubers from room D in the interval from 29 days to 60 days in cold storage, the increase for this room being only about 60 per cent of the increase for the other rooms during this interval.

While the effect of the pre-storage condition upon the increase in reducing sugar during cold storage was not large, the result indicates that storage at the lower temperature induced the tubers to reach a lower final reducing sugar value. The result is not entirely conclusive and further tests would be required. Possibly temperatures lower than 15° C. would be more effective provided they were not such as to cause an increase in the reducing sugar during the pre-storage period itself.

Sucrose. The results for sucrose are shown in Table II. As shown in columns 3, 7, 11, and 15, there were appreciable amounts of sucrose in the juice of the tubers at the end of each pre-storage interval at the time of transfer to cold storage, so that the values in Table II show the actual sucrose values at each sampling period, and not the gains during the intervals, as was the case with the reducing sugar values in Table I. For the analysis of variance for sucrose (Table III, columns 6 to 11), a

TABLE I

EFFECT OF POST-HARVEST PRE-STORAGE CONDITIONS ON THE RATE OF DEVELOPMENT OF REDUCING SUGAR IN POTATO TUBERS DURING SUBSEQUENT COLD STORAGE

Days in cold storage	Date of start of cold storage	Gain in reducing sugar, mg./cc. of juice of tubers stored at 5° C.															
		Room A Temp. = 23.3° C.* Humid. = 42%*				Room B Temp. = 24.4° C.* Humid. = 92%*				Room C Temp. = 19.8° C.* Humid. = 70%*				Room D Temp. = 15° C.* Humid. = 86%*			
		Variety**				Variety**				Variety**				Variety**			
		G.M.	Car.	Cob.	Kat.	G.M.	Car.	Cob.	Kat.	G.M.	Car.	Cob.	Kat.	G.M.	Car.	Cob.	Kat.
15	Oct. 1	4.1	0.0	2.8	0.2	4.2	1.5	3.4	1.2	4.7	2.9	5.8	2.4	6.3	0.9	4.2	3.4
	Oct. 26	1.3	0.0	4.1	1.3	1.0	0.0	1.8	2.2	1.5	0.1	3.6	3.3	3.8	0.0	4.1	2.1
	Dec. 1	0.9	0.0	0.8	0.0	0.9	0.0	0.0	0.0	0.9	0.0	0.8	2.7	2.3	0.0	0.6	3.9
	Dec. 24	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	2.3	0.0	0.1	2.3	0.3	0.2	1.5	3.4
Room total		16.5				16.9				33.9				37.0			
29	Oct. 1	10.5	4.6	8.3	7.6	10.2	4.8	11.4	9.7	8.2	4.3	13.4	6.5	10.3	2.6	10.0	9.7
	Oct. 26	9.6	4.9	7.8	5.9	8.2	6.8	10.9	10.1	6.8	3.3	10.4	7.6	8.7	2.3	7.7	9.3
	Dec. 1	7.3	2.2	2.3	3.4	6.4	2.9	2.6	7.0	5.4	0.0	7.0	7.9	3.6	0.5	4.0	6.0
	Dec. 24	5.6	0.8	4.3	4.3	4.6	1.6	0.1	6.2	4.9	0.0	4.2	4.1	2.9	0.0	4.9	5.3
Room total		89.4				103.5				94.0				87.8			
60	Oct. 1	12.7	4.8	12.3	11.9	13.9	4.9	13.4	12.6	11.4	8.7	19.6	12.7	8.9	2.3	11.0	14.7
	Oct. 26	17.8	5.4	15.5	11.5	16.2	6.8	15.9	14.9	9.1	7.2	14.1	13.7	10.2	2.5	10.3	10.1
	Dec. 1	10.4	2.3	9.6	7.0	8.6	3.0	9.1	10.1	7.0	1.2	9.2	9.0	5.7	0.8	6.8	7.9
	Dec. 24	10.0	0.4	4.1	10.5	7.6	2.3	4.8	10.5	6.5	0.1	8.1	9.2	8.1	0.0	9.7	7.7
Room total		146.2				154.6				146.8				116.7			

* Conditions of storage in the interval from shortly after harvest until tubers were placed in cold storage at 5° C., starting at dates shown in column 2.

** The abbreviations correspond to the varieties: Green Mountain, Carman No. 3, Irish Cobbler, and Katahdin.

TABLE II

EFFECT OF POST-HARVEST PRE-STORAGE CONDITIONS ON THE RATE OF DEVELOPMENT OF SUCROSE IN POTATO TUBERS DURING SUBSEQUENT COLD STORAGE

Pre-storage condition*	Variety**	Milligrams of sugar per cc. of juice of tubers stored at 5° C.															
		Storage started Oct. 1				Storage started Oct. 26				Storage started Dec. 1				Storage started Dec. 24			
		Days of storage				Days of storage				Days of storage				Days of storage			
		0	15	29	60	0	15	29	60	0	15	29	60	0	15	29	60
Room A Temp. = 23.3° C. 73.9° F. Humid. = 42%	G.M.	3.8	15.3	14.5	8.7	2.5	17.3	13.0	9.0	1.7	17.8	14.2	12.0	1.9	20.0	16.3	14.7
	Car.	2.0	11.2	10.3	3.2	1.4	8.1	4.9	4.5	0.4	4.5	4.3	2.5	0.4	4.2	4.5	3.5
	Cob.	2.0	9.7	16.5	3.9	0.6	8.5	9.9	7.6	0.6	13.0	15.7	8.9	0.6	17.8	15.8	19.4
	Kat.	1.9	8.9	7.9	4.2	3.5	10.6	6.0	3.6	2.7	8.4	8.9	7.0	1.7	11.6	10.5	11.7
Room B Temp. = 24.4° C. 75.9° F. Humid. = 92%	G.M.	3.7	14.9	16.2	8.1	3.0	18.4	16.5	13.4	4.0	20.2	18.4	17.6	2.5	21.4	23.3	16.7
	Car.	2.2	11.5	8.6	3.2	1.0	8.2	4.3	2.4	1.2	4.3	6.3	2.9	0.4	7.4	4.7	2.5
	Cob.	2.0	11.5	7.3	3.5	0.7	14.3	7.4	4.7	1.6	14.0	19.2	13.6	1.4	21.0	27.1	24.7
	Kat.	1.5	11.5	5.4	4.3	1.4	9.2	5.8	2.2	2.2	9.7	6.8	6.9	3.0	11.1	8.1	7.0
Room C Temp. = 19.8° C. 67.6° F. Humid. = 70%	G.M.	4.2	18.9	23.8	12.2	2.6	21.6	21.0	17.6	2.7	15.4	16.9	12.9	1.9	14.4	12.7	13.3
	Car.	1.1	11.7	12.2	4.7	0.6	9.1	8.5	5.4	0.1	3.8	5.0	3.1	0.0	3.7	4.4	3.3
	Cob.	2.3	12.0	13.4	7.2	1.2	15.1	13.7	9.3	0.7	10.8	14.0	10.0	1.6	14.4	11.3	9.6
	Kat.	1.6	12.9	15.9	6.5	0.4	13.7	11.9	6.6	0.2	6.1	7.5	4.4	1.0	7.7	8.0	8.1
Room D Temp. = 15° C. 59° F. Humid. = 86%	G.M.	3.6	7.8	9.2	6.0	3.4	12.6	11.3	6.5	3.3	7.4	7.9	5.1	2.4	8.5	8.4	7.6
	Car.	2.4	5.9	5.3	3.8	0.2	8.5	4.6	3.3	0.4	1.8	4.7	2.2	0.1	2.3	3.0	3.1
	Cob.	0.5	6.9	5.9	4.0	1.6	4.9	5.4	3.1	0.9	5.6	6.9	4.4	0.4	6.4	7.1	6.3
	Kat.	1.6	6.9	5.6	3.8	0.8	9.8	4.5	2.6	0.7	3.7	2.0	2.7	0.2	4.9	3.8	4.6

* Conditions of storage in the interval from shortly after harvest until tubers were placed in cold storage at 5° C. (41° F.).
** The abbreviations correspond to the varieties: Green Mountain, Carman No. 3, Irish Cobbler, and Katahdin.

TABLE III

ANALYSIS OF VARIANCE OF VALUES IN TABLES I AND II

Reducing sugar values in Table I					Gain in sucrose during storage intervals, in Table II					
Source of variation	D.F.	Variance for three storage intervals			Source of variation	D.F.	Variance for the varieties*			
		15 days	29 days	60 days			G.M.	Car.	Cob.	Kat.
Storage rooms	3	7.42	3.11	14.08	Storage rooms	3	210.90	12.87	143.92	42.95
Starting dates	3	17.46	91.86	117.71	Starting dates	3	27.55	16.67	129.58	7.88
Varieties	3	12.12	75.49	218.24	Days of storage	2	92.68	48.36	56.08	57.29
Rooms × Start.	9	0.44	1.47	6.10	Rooms × Start.	9	19.83	4.22	38.25	11.56
Rooms × Var.	9	1.30	6.62	6.98	Rooms × Days	6	4.40	2.74	10.96	5.03
Start. × Var.	9	3.71	3.93	3.45	Start. × Days	6	6.68	9.20	17.54	9.50
R. × S. × V.	27	2.70	1.56	2.88	R. × S. × D.	18	1.95	1.08	1.11	1.15

* The abbreviatons correspond to the varieties: Green Mountain, Carman No. 3, Irish Cobbler, and Katahdin.

separate table (not shown here) was prepared in which the gains in sucrose during the 15-, 29-, and 60-day intervals for each starting date were entered.

Tables II and III show that the increase in sucrose was much less in tubers that had been pre-stored at 15° than in tubers from the other rooms. This is true of all of the varieties and with tubers at all starting dates. The gain in sucrose was approximately one-half as much with tubers pre-stored in room D, as in those stored in rooms A, B, and C.

Humidity. A test of the effect of humidity was available only by the comparison of rooms A and B. In these, although the temperature was nearly the same, the humidity was low in room A, 42 per cent, and high in room B, 92 per cent. The results from these two rooms were quite similar, however, and indicate that at that temperature the humidity condition during the pre-storage period had little effect on the capacity of the tubers to form sugar in subsequent cold storage.

SUMMARY

Potato tubers (*Solanum tuberosum* L.) of four varieties were placed on August 27 soon after harvest under four different conditions of pre-storage to determine the effect of post-harvest storage conditions upon the sugar changes in tubers subsequently transferred to cold storage.

The pre-storage conditions were as follows: high temperature (23.3° to 24.4° C.) and low humidity (42 per cent); high temperature (23.3° to 24.4° C.) and high humidity (92 per cent); medium temperature (19.8° C.) and a humidity of 70 per cent; low temperature (15° C.) and a humidity of 86 per cent.

On October 1, October 26, December 1, and December 24, samples of tubers were removed from these pre-storage conditions and placed in cold

storage at 5° C. At intervals of 15, 29, and 60 days thereafter samples were removed for sugar analyses.

The principal effect of the pre-storage conditions was upon the sucrose content during subsequent cold storage. Tubers pre-stored at 15° C. developed only about one-half as much sucrose as tubers pre-stored under the other three storage conditions. There was also some evidence that the level of reducing sugar formed during cold storage was lowered by about 20 per cent as a result of pre-storage at 15° C.

The post-harvest pre-storage conditions did not influence the sugar content of the tubers during the pre-storage period. The effects found were upon the sugar contents of the tubers after they were transferred to cold storage at 5° C.

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THE EFFECT OF LOW CONCENTRATIONS OF CARBON DIOXIDE UPON THE SUGAR CONTENT OF POTATO TUBERS IN COLD STORAGE

F. E. Denny and Norwood C. Thornton

It was shown in previous reports (2, 3) that the addition of carbon dioxide to the atmosphere surrounding potato tubers in cold storage at about 5° C. prevented the rapid increase in reducing sugar which occurs at that temperature, and at the same time increased the amount of sucrose.

The lowest concentration of CO_2 used in the previous tests was 5 per cent by volume, this amount being effective in all cases. The object of the present experiments was to test the concentration interval between 0 and 5 per cent.

It was found that decreasing the amount of CO_2 to 2.49 per cent reduced the effectiveness by only about 20 per cent, but that a still further reduction of the CO_2 to 1.27 per cent by volume was critical, and indicated that 1 per cent CO_2 was close to the marginal concentration.

This was confirmed by a subsequent experiment in which 1.1 per cent CO_2 was used. This amount had a definite influence, holding the reducing sugar to lower values than those of the controls, and increasing the amount of sucrose. This condition held during an interval of about 40 days from the start of the test. By the end of 50 days, however, the values for the CO_2-treated and the control lots were nearly the same.

At the end of the 30-day duration period, the lots receiving 1.1 per cent CO_2 showed a reducing sugar increase amounting to about 71 per cent of that of the control without CO_2, and a sucrose increase about 124 per cent of the control.

MATERIALS AND METHODS

Containers and CO_2 concentrations. Galvanized iron cylinders each of 790 liters capacity were used as containers for maintaining the desired concentrations of carbon dioxide. These were placed within a cold storage room which was thermostatically adjusted at 5° C. The temperature was observed several times daily by the storage room attendants, and adjustments of the thermostat made as needed. The CO_2 concentrations were maintained at the desired level by gas analysis of a sample of air from each container and by adjustment with CO_2 from a cylinder of the compressed gas. After each adjustment another sample was taken to make certain that the proper correction had been made. The volume of a con-

tainer was approximately 30 times that of the tuber samples. The CO_2 concentrations listed in the tables are the time-weighted averages of the CO_2 values obtained before and after each adjustment. The concentration of O_2 was maintained at 20 per cent by volume, or slightly higher, continuously.

Potato tubers. The tubers (*Solanum tuberosum* L.) were obtained from a local wholesale market, and were assorted into sizes which were distributed evenly into samples of 20 tubers each, tied in cheesecloth bags. Triplicate samples were taken at each removal interval in the 0 to 5.0 per cent CO_2 series, and duplicates in the test with 1.1 per cent CO_2.

Sugar analyses. At each sampling period the tubers were washed, dried, and minced in a food grinder. The juice was obtained by squeezing the tissue in a cheesecloth bag, was centrifuged, and 25-cc. samples were taken for analyses. The analyses for reducing sugar and sucrose were carried out as previously described (1, p. 294).

RESULTS

EFFECT OF CO_2 IN THE CONCENTRATION RANGE: 0 TO 5 PER CENT

The results of this test are shown in Table I. The tubers available for this experiment were of the variety Irish Cobbler. A sugar analysis of this lot at the start of storage showed no reducing sugar (or at least less than 0.1 mg.) and 2.8 mg. of sucrose per cc. of juice. The values in Table I are the gains made from this base during the storage period. The three values in each cell of Table I are those shown by the triplicate samples of tubers removed at each sampling interval.

It was intended that the CO_2 concentrations within the containers should be 1.25, 2.5, and 5.0 per cent CO_2 (by volume). However, the averages of the day-by-day gas analyses showed that the concentrations actually employed were 1.27, 2.49, and 4.90 per cent, and these are the amounts entered in the heading of Table I.

Table I shows that 4.90 and 2.49 per cent CO_2 were quite effective in retarding the rate of increase in reducing sugar and in increasing that of sucrose. The reducing sugar increase (columns 2 to 5) was held to about 45 and 35 per cent of that of the control rate by 2.49 and 4.90 per cent, respectively. The reducing sugar values reached by the control lot at the 15-day sampling period were not reached by the 4.90 per cent CO_2 lot until 45 days after the start of storage, or until 30 days by the lot receiving 2.49 per cent CO_2. There was about a 15-day lag between the lots with 1.27 per cent and 2.49 per cent.

The sucrose increases (columns 6 to 9) were much greater for the CO_2-treated lots, the gain at the end of the 45-day period being more than four

times as great for 2.49 and 4.90 per cent CO_2 as for the control lot. By the thirtieth day the sucrose gain in the lot receiving 1.27 per cent became about twice that of the control lot, and this difference was maintained to the end of the experiment.

If one wishes to deal with actual amounts of sucrose per cc. of juice at each interval rather than with increases, these values may be obtained by adding 2.8 (the starting value) to each of the sucrose values in Table I.

TABLE I

EFFECT OF CONCENTRATION OF CO_2 IN THE RANGE 0 TO 5 PER CENT UPON THE SUGAR CONTENT OF POTATO TUBERS STORED AT 5° C.

No. of days in cold storage at 5° C.	Gain in sugar, mg. per cc. of juice							
	Reducing sugar				Sucrose			
	Amt. of CO_2, % by volume				Amt. of CO_2, % by volume			
	No CO_2	1.27%* CO_2	2.49%* CO_2	4.90%* CO_2	No CO_2	1.27%* CO_2	2.49%* CO_2	4.90%* CO_2
15	2.7 2.8 3.3	2.3 2.3 2.0	1.7 1.2 1.9	1.3 1.8 1.2	6.8 6.9 6.8	9.7 10.3 10.5	12.2 12.9 12.9	14.9 14.6 15.5
30	6.6 6.5 6.1	5.3 4.9 4.9	2.4 3.7 3.4	1.7 2.1 1.6	5.9 6.6 6.2	13.7 13.6 13.4	16.4 16.4 15.2	18.1 18.5 19.1
45	9.6 10.1 10.1	8.6 8.5 8.3	3.9 4.3 4.0	2.6 3.1 3.4	4.1 4.6 4.8	10.7 11.3 12.9	18.4 17.9 17.7	21.2 19.5 19.7
Totals	57.8	47.1	26.5	18.8	52.7	106.1	140.0	161.1

* The standard deviations of the means 1.27, 2.49, and 4.90 were 0.005, 0.005, and 0.014 respectively; $n=35$.

Notes: (a) Tubers were of the variety Irish Cobbler, and showed sugar contents of juice at start of cold storage as follows: reducing sugar none, sucrose 2.8 mg./cc.
(b) The three values in each cell of the table are the values corresponding to triplicate samples of tubers.

This shows that the control lot showed a maximum sucrose content at at the end of the 15-day interval, and then decreased progressively to the end of the test, while the 2.49 and 4.90 per cent CO_2 lots showed continuous increases in sucrose, the final values being the highest. The lot receiving 1.27 per cent CO_2 was intermediate in behavior between these showing a maximum sucrose value at the 30-day interval, which was followed by a decrease on further storage.

The reducing sugar gains for the lots receiving 1.27 per cent CO_2 are considerably higher than those for 2.49 per cent and approach more closely those of the control lot. The difference between the control lot and that with 1.27 per cent CO_2 is greater than the error among the

replicates in these two columns, but we cannot be sure that the difference between potato sample replicates is a dependable measure of error in the experiment, because there is doubt as to the error between two different containers with the same concentration of CO_2. In the experiment now to be described, this question was dealt with by having two containers at the same CO_2 concentration, and then comparing the differences found between CO_2 concentrations with the differences between duplicate containers at the same concentration.

EFFECT OF 1.1 PER CENT CO_2

Since the previous experiment had shown that there was a distinct reduction in the CO_2 effect at 1.27 per cent CO_2, a test was made at approximately 1 per cent CO_2 to determine whether a difference between no CO_2 and 1 per cent CO_2 could be shown. The storage room space was of such size as to accommodate only four of the galvanized containers. In order to determine the error not only between potato samples in the same container but also between two containers with the same CO_2 treatment, two of the containers were used for the CO_2 treatment, and two for the control without CO_2. Although it was intended that the amount of CO_2 should be 1 per cent, the average amounts actually found during the experiment by the day-by-day gas analyses were 1.10 and 1.09 per cent CO_2 in the two containers assigned to CO_2 treatment. Gas analyses were made of the control containers at intervals to make certain that there was no CO_2 in the air in them.

The tubers available for this test were of the variety Bliss Triumph. Since, as received, these tubers showed considerable sugar in the juice, they were allowed to stand at room temperature for a preliminary period in order to bring about a reduction in the sugar content. It was found, however, that de-sugaring occurred in this lot quite slowly, and practically ceased at the end of three weeks.

Therefore, at the time the cold storage and CO_2 treatments were started, this lot showed 2.3 mg. of reducing sugar and 4.8 mg. of sucrose per cc. of juice. The values shown in Table II are the gains in sugar above these values as a base.

Samples were removed at intervals of 10, 20, 30, 40, and 50 days after the start of cold storage at 5° C. (Table II, column 1). There were two control containers (A and B in Table II, columns 2, 3, 6, and 7), and two with added CO_2 (C and D in Table II, columns 4, 5, 8, and 9). The paired values in each of the cells of the table are those from the duplicate samples of potatoes taken from each container at each removal interval.

The analysis of variance of the data for this experiment is shown at the bottom of Table II. The error term was considered to be one in the last line of the table, i.e., the variance between duplicate containers at each removal interval, 0.60 for reducing sugar and 1.41 for sucrose.

TABLE II

EFFECT OF 1.1 PER CENT CO_2 (BY VOLUME) UPON THE SUGAR CONTENT OF POTATO TUBERS STORED AT 5° C.

No. of days in cold storage at 5° C.	Gain in sugar, mg. per cc. of juice							
	Reducing sugar				Sucrose			
	No CO_2 Container		1.1% CO_2 Container		No CO_2 Container		1.1% CO_2 Container	
	A	B	C*	D*	A	B	C*	D*
10	2.5 2.7	2.9 3.1	2.4 2.8	2.6 2.8	3.9 2.8	2.8 3.5	3.8 4.1	4.4 5.0
20	6.0 6.1	7.1 6.8	5.7 4.0	4.2 4.9	10.9 11.5	10.6 9.7	13.2 13.6	13.4 11.5
30	10.6 9.9	11.2 9.7	7.7 8.1	6.4 7.2	11.5 11.9	10.4 11.5	14.5 13.3	14.8 13.5
40	14.9 13.3	14.0 12.3	11.1 11.1	10.9 10.8	5.6 8.4	8.9 9.6	12.1 12.1	11.6 10.4
50	16.1 16.2	18.3 16.7	17.1 15.2	14.7 15.7	5.4 4.8	4.4 6.0	5.0 7.1	9.7 6.7
Totals	98.3	102.1	85.2	80.2	76.7	77.4	98.8	101.0

* Average concentration of CO_2 was 1.10% by volume for C, and 1.09% for D. The standard deviations of the means 1.10 and 1.09 were 0.005 and 0.006 respectively; $n=43$.

Note: Tubers were of the variety Bliss Triumph, and showed sugar contents of juice at start of cold storage as follows: reducing sugar 2.3, sucrose 4.8 mg. per cc. of juice.

Analysis of Variance of Data in Above Table

Source of variation	Degrees of freedom	Variance	
		Reducing sugar	Sucrose
CO_2 concentrations	1	30.63	52.21
Days duration	4	228.61	114.59
$CO_2 \times$ Days	4	2.65	1.52
Duplicate samples of tubers	20	0.50	0.94
Container replicates at each removal date	10	0.60	1.41

The F values (4, p. 183) for the over-all CO_2 effect with both reducing sugar and sucrose, 51 and 37 respectively, were highly significant, being well beyond the 0.01 point. The time of occurrence of the largest difference between the controls and 1.1 per cent CO_2 was at the 30- and 40-day intervals after the start. At that time the reducing sugar content of the CO_2 lot was about 20 per cent lower than that of the control, while the sucrose content was about 20 per cent higher.

After the fortieth day, however, the curves for the CO_2 lots began to approach those of the controls, so that on the fiftieth day the reducing

sugar values were not conclusively different, at least not with odds of 19 to 1. The F value (4, p. 339) for the reducing sugar comparison of the two lots at the fiftieth day may be computed as follows: $F=[(16.1+16.2+18.3+16.7)-(17.1+15.2+14.7+15.7)]^2 \div (8\times 0.60)=4.41$. This is slightly less than 4.96, which is the required F value for a probability of 0.05 with 1 and 10 degrees of freedom.

However, the sucrose values remain different even on the fiftieth day. The F value for sucrose comparison at that time, computed in the manner illustrated above (using 1.41 instead of 0.60), is 5.53, which indicates odds of better than 19 to 1 that the sucrose gain for the treatment with 1.1 per cent CO_2 was greater than that for the control even at the fiftieth day.

The F values for similar comparisons on the thirtieth and fortieth days are 30.0 and 23.4 for reducing sugar; 10.3 and 16.6 for sucrose—showing large differences between the lots with 1.1 per cent CO_2 and the corresponding controls, all F values being higher than that required for odds of 99 to 1.

SUMMARY

The increase in sugar content which occurs when potato (*Solanum tuberosum* L.) tubers are placed in cold storage at 5° C. was influenced by adding small amounts of carbon dioxide to the atmosphere surrounding the tubers.

The effect of the CO_2 was to retard the development of reducing sugar and increase that of sucrose. Over a period of 45 days the increase in reducing sugar in the presence of 4.90 per cent CO_2 was only about one-third as much as that of the control without CO_2, and about two-fifths as much with a CO_2 concentration of 2.49 per cent.

The lowest concentration tried, 1.1 per cent CO_2, had a definite effect, retarding the rate of increase of reducing sugar by about 20 per cent and increasing the gain in sucrose by about 30 per cent during a storage interval of 30 to 40 days.

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TOXICITY OF ORGANIC COMPOUNDS TO HOUSEFLIES

Edward K. Harvill and John M. Arthur

The need for synthetic organic insecticides has been emphasized by the insufficiency of the naturally occurring pyrethrum and rotenone compounds. The shortage of pyrethrum products is especially serious for the preparation of household fly sprays, and, although recent work has resulted in the development of activators which have the property of enhancing the toxicity of the pyrethrins and so extending their use, very few compounds have the rapid paralyzing action necessary to replace the pyrethrins entirely.

Several years ago a phase in the investigation of toxicants for the control of houseflies had been completed and compounds having both a rapid paralyzing effect and appreciable toxicity had been prepared. In an attempt to secure information as to any correlation between toxicity and structure a survey was made of the effect of various naturally occurring compounds on houseflies. Of the substances tested, several essential oils were found to have fairly good toxicity and paralyzing action although only at high concentrations. The presence of a nuclear allyl group in all these compounds suggested that the synthesis of different allyl phenols might produce materials with insecticidal action. The results secured with isomeric eugenols, allyl phenols, and substituted allyl phenols verified this.

In order to increase further the toxicity of these various phenols the γ-thiocyanopropyl and β-thiocyanoethyl ethers of these phenols and of the cresols and 1,3,5-xylenol were made. These thiocyanates had exceptional toxicity and paralyzing action and were well suited for use in household fly sprays. The LD50s of these compounds varied from 0.63 to 1.24 per cent.

TOXICITY EXPERIMENTS

The compounds were tested on houseflies (*Musca domestica* L.) by the large Peet-Grady method following exactly the procedure recommended by the National Association of Insecticide and Disinfectant Manufacturers (5). The toxicity curve for each compound was determined by testing at least six samples at different concentrations. In compounds of particular interest as many as 20 tests were used to determine the curve. The results of the toxicity tests were adjusted by means of probit values so that the standard pyrethrum solution containing 100 mg. of pyrethrins in 100 cc. of "Deo-base" gave a 50 per cent kill. The dosage-mortality curve was then calculated according to the procedure outlined by Bliss (1) and the LD50

for each compound was calculated from the formula and expressed as per cent. The error was the standard deviation of X. In this way the LD50 of all the compounds are comparable and are based on a kill of 50 per cent for the standard pyrethrum solution. The formula for the dosage-mortality curves is given in Tables II to VIII.

The spray solutions were made by dissolving the compounds in "Deo-base," a highly refined kerosene. Since some of the compounds, especially the thiocyanates, were insoluble in "Deo-base," acetone was used to form a homogeneous solution.

PREPARATION OF COMPOUNDS

Allyl ethers of phenols. The allyl ethers of the phenols were made by refluxing an alcoholic solution containing equivalent amounts of the appropriate phenol, sodium ethoxide, and allyl bromide until the reaction mixture was neutral to litmus paper. The mixture was strongly diluted with water and the oily layer separated, washed with 10 per cent sodium hydroxide and then with water, and dried over anhydrous sodium sulphate. Vacuum distillation gave the allyl ethers in 60 to 80 per cent yield.

Allyl phenols. The allyl phenols have been made by Claisen by rearrangement of the allyl ethers (2, 3, 4).

The isomeric eugenols were made by rearranging the allyl methyl ethers of catechol, resorcinol, and hydroquinone to 2-allyl-6-methoxyphenol, 2-allyl-5-methoxyphenol, and 2-allyl-4-methoxyphenol.

γ-Thiocyanopropyl and β-thiocyanoethyl ethers of phenols. The thiocyanogen compounds were prepared in the usual way by refluxing the γ-brompropyl or β-bromethyl ethers in alcohol with an excess of potassium thiocyanate.

The γ-brompropyl and β-bromethyl ethers were made in the same manner as the allyl ethers of the phenols using trimethylene bromide and ethylene bromide instead of allyl bromide.

DISCUSSION OF RESULTS

The results of toxicity tests of eugenol, safrol, isosafrol, and anethole are given in Table I, and similar results were obtained with isoeugenol, and with the allyl and methyl ethers of isoeugenol and eugenol.

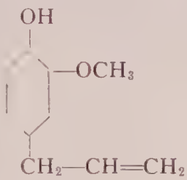
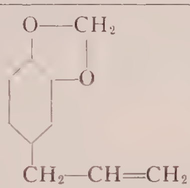
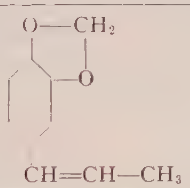
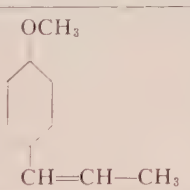
Further tests were made with the isomeric eugenols (Table II) and the results showed a great variation in toxicity for these isomers. 2-Allyl-4-methoxyphenol and 4-allyl-2-methoxyphenol (eugenol) were about equally toxic, interchange of the allyl and methoxyl groups in the 2 and 4 position causing no great change in toxicity. 2-Allyl-6-methoxyphenol at concentrations up to 20 per cent had a very slight effect on flies. The knockdown and kill were too small to allow a determination of the dosage-mortality curve. The most toxic member of the groups was 2-allyl-5-methoxyphenol which

had a median lethal dose of 4.76 per cent. Any effect of the allyl group was either enhanced or inhibited by the introduction of a methoxyl group, substitutions in the 2 and 5 position producing the most favorable joint effect.

Whether the introduction of an allyl group into the nucleus would increase the insecticidal activity of a compound was determined by testing

TABLE I

TOXICITY OF ALLYL AND PROPENYL COMPOUNDS TO FLIES
(LARGE PEET-GRADY METHOD)

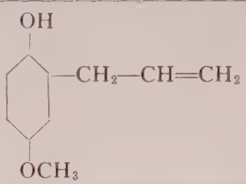
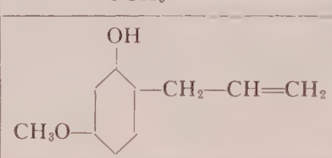
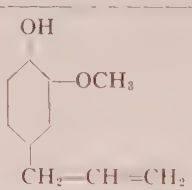
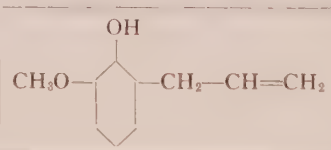
Compound	Structure	Per cent		
		Concentration	Knockdown	Kill
Eugenol		20.0	96.0	57.2
Safrol		20.0	99.0	25.0
Isosafrol		20.0	95.9	73.0
Anethole		20.0	96.1	49.3

the simple allyl phenols to eliminate any joint effects such as occurred with the isomeric eugenols. Phenol at concentrations up to 20 per cent gave no knockdown and less than 5 per cent kill in 24 hours in a fly test. This was determined by keeping the flies in the test chamber for 24 hours after spraying with the phenol solution. *o*-Allylphenol gave a definite knockdown of 90 to 95 per cent at concentrations over 15 per cent and had a

median lethal dose of 18.5 per cent. Increasing the number of allyl groups increased the knockdown and kill. The diallyl- and triallylphenols gave over a 95 per cent knockdown in ten minutes at their median lethal doses of 10.5 and 2.63 per cent (Table III). The addition of an allyl group increased insecticidal activity. Further additions increased the paralytic ef-

TABLE II

TOXICITY OF ISOMERIC EUGENOLS TO FLIES

Compound	Structure	Dosage-mortality formula*	LD50 per cent
2-Allyl-4-methoxyphenol		$Y=4.8850+1.0762(X-0.8639)$	9.35
2-Allyl-5-methoxyphenol		$Y=4.9411+1.8301(X-0.6745)$	4.76
4-Allyl-2-methoxyphenol		$Y=5.1613+2.5242(X-1.1244)$	11.49
2-Allyl-6-methoxyphenol		**	**

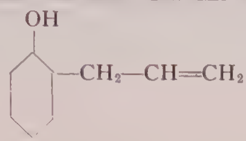
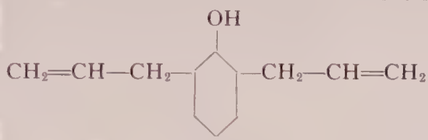
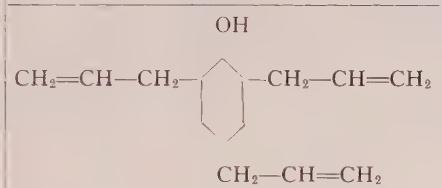
* Y = Probit value; X = Log. (concentration).
** Toxicity and knockdown too poor to allow determination of dosage-mortality curve.

fect and about doubled the toxicity of the compound with the introduction of each additional group.

The *o*-allylphenols containing an *o*-chloro, *p*-chloro, *p*-bromo, *o*-nitro, or *o*-methyl group were of the same order of toxicity as the *o,o'*-diallylphenol. However, methyl substitutions in the meta and para position of *o*-allylphenol produced changes in toxicity similar to those found in the isomeric eugenols (Table IV). *o*-Methyl-, *m*-methyl-, and *p*-methylphenol were non-toxic to flies. *o*-Allyl-*o'*-methylphenol was least toxic and *o*-allyl-*m*-methylphenol most toxic of the allyl cresols.

TABLE III

TOXICITY OF ALLYL PHENOLS TO FLIES

Phenol	Structure	Dosage-mortality formula*	LD50 per cent
o-Allyl		Y=4.9767+2.9622 (X—1.2691)	18.5 ±1.8
o,o'-Diallyl		Y=5.1667+2.3412 (X—1.0934)	10.5 ±0.59
o,o', p-Triallyl		Y=5.8383+2.2227 (X—0.7975)	2.63 ±1.3

* Y=Probit value; X=Log. (concentration).

The results show that non-toxic phenols by the introduction of a nuclear allyl group develop insecticidal properties. An increase in toxicity may then be effected by the introduction of another allyl group. However, the introduction of a methyl or methoxyl group in the meta position increases the toxicity of *o*-allylphenol much more than the addition of another allyl group in the ortho position.

The greater toxicity of 2,6-diallylphenol (LD50 10.5 per cent) over that of 2-allyl-6-methoxyphenol which has very little insecticidal value suggests that 2,5-diallylphenol should be much more effective than 2-allyl-5-methoxyphenol (LD50 4.76 per cent).

The use of the allyl phenols as insecticides presents several disadvantages. Even the more toxic compounds have a slow knockdown compared

TABLE IV

TOXICITY OF ALLYL PHENOLS TO FLIES

Compound	LD50 per cent
o-Allyl-*o*-cresol	13.93±0.54
o-Allyl-*m*-cresol	5.05±0.60
o-Allyl-*p*-cresol	6.55±0.12
o,o'-Diallylphenol	10.5 ±0.59

to the pyrethrins. These phenols, except for the naturally occurring essential oils, have a penetrating and disagreeable odor which is intensified when sprayed as a mist.

Compounds having a more rapid paralyzing effect and a more agreeable odor were secured by masking the free phenolic group with a substituent which contained a thiocyanogen group. The ethers of the phenols were found to have a less irritating odor and the thiocyanogen group increased the toxicity greatly.

TABLE V

TOXICITY OF GAMMA THIOCYANOPROPYL ETHERS OF METHOXYALLYL PHENOLS TO FLIES

Phenol	Toxicity formula*	LD50 per cent
4-Allyl-2-methoxy	$Y = 5.4703 + 2.0612\ (X - 1.1131)$	0.76 ± 0.07
2-Allyl-4-methoxy	$Y = 5.3646 + 3.3203\ (X - 1.0491)$	0.87 ± 0.03
2-Allyl-6-methoxy	$Y = 5.1230 + 2.3904\ (X - 1.0080)$	0.90 ± 0.06
2-Allyl-5-methoxy	$Y = 5.4667 + 1.9315\ (X - 1.0204)$	0.60 ± 0.06
4-Propenyl-2-methoxy	$Y = 5.3128 + 2.0931\ (X - 1.1407)$	1.33 ± 0.18

* Y = Probit value; X = Log. (10) (concentration).

TABLE VI

TOXICITY OF GAMMA THIOCYANOPROPYL ETHERS OF PHENOLS TO FLIES

Phenol	Toxicity formula*	LD50 per cent
o-Allyl	$Y = 5.1507 + 2.5358\ (X - 1.1679)$	1.28 ± 0.08
o,o'-Diallyl	$Y = 5.3948 + 2.1560\ (X - 1.1020)$	0.96 ± 0.18
o,o', p-Triallyl	$Y = 5.4083 + 2.4357\ (X - 1.0132)$	0.70 ± 0.03
o-Allyl-*o*-methyl	$Y = 5.3407 + 2.0373\ (X - 0.9698)$	0.63 ± 0.24
o-Allyl-*m*-methyl	$Y = 5.6265 + 2.5578\ (X - 1.0693)$	0.67 ± 0.14
o-Allyl-*o*-chlor	$Y = 5.5185 + 2.2498\ (X - 1.1400)$	0.81 ± 0.08
o-Allyl-*p*-chlor	$Y = 5.3513 + 2.6231\ (X - 1.1766)$	1.10 ± 0.10
o-Allyl-*o'*, *p*-dichlor	$Y = 5.3591 + 2.2948\ (X - 1.1246)$	0.92 ± 0.04
o-Allyl-salicylaldehyde	$Y = 5.4313 + 2.6033\ (X - 1.1201)$	0.90 ± 0.09
o-Allyl-*p*-brom	$Y = 5.5116 + 2.2541\ (X - 1.0217)$	0.69 ± 0.09

* Y = Probit value; X = Log. (10) (concentration).

The tests with the γ-thiocyanopropyl ethers of the isomeric eugenols (Table V) showed the same variation in toxicity as the phenols. The most toxic compound was the γ-thiocyanopropyl ether of 2-allyl-5-methoxyphenol which had an LD50 of 0.60 per cent. This increase in toxicity was paralleled by an increased rapidity in knockdown that compared very favorably with the pyrethrins. The effectiveness of the substituted allyl phenols was increased similarly (Table VI) and where the most toxic of the allyl phenols, triallylphenol, had an LD50 of 2.63 per cent the median lethal dose of the γ-thiocyanopropyl ethers varied between 1.33 and 0.60 per cent.

The relatively small change in toxicity with various nuclear substitutions of the γ-thiocyanopropyl ethers was apparent in the results secured with the ethers of phenol, ortho, meta, and para cresol and 1,3,5-xylenol (Table VII). There was very little difference in toxicity and knockdown between these compounds and the γ-thiccyanopropyl ethers of the allyl phenols.

The most suitable compound of the group for household fly sprays was

TABLE VII

TOXICITY OF GAMMA THIOCYANOPROPYL ETHERS OF PHENOLS TO FLIES

Phenol	Toxicity formula*	LD50 per cent
Phenol	$Y=4.9913+3.4646\ (X-1.0106)$	1.03 ± 0.02
o-Methyl	$Y=5.5203+2.3809\ (X-1.0847)$	0.73 ± 0.04
m-Methyl	$Y=4.8964+5.5355\ (X-0.8274)$	0.70 ± 0.08
p-Methyl	$Y=5.3715+3.1666\ (X-0.9182)$	0.63 ± 0.05
m,m'-Dimethyl	$Y=5.3514+2.4551\ (X-1.0410)$	0.79 ± 0.04

* Y = Probit value; X = Log. (10) (concentration).

TABLE VIII

TOXICITY OF BETA THIOCYANOETHYL ETHERS OF PHENOLS TO FLIES

Phenol	Toxicity formula*	LD50 per cent
o-Methyl	$Y=4.8894+2.3908\ (X-1.0473)$	1.24 ± 0.03
m-Methyl	$Y=5.3223+2.3741\ (X-1.0828)$	0.89 ± 0.06
p-Methyl	$Y=5.2174+2.6308\ (X-0.9928)$	0.81 ± 0.05
m,m'-Dimethyl	$Y=5.4584+2.7483\ (X-1.0169)$	0.70 ± 0.06

* Y = Probit value; X = Log. (10) (concentration).

the γ-thiocyanopropyl ether of 1,3,5-xylenol. It had an LD50 of 0.79 per cent and a very rapid knockdown effect. Most important for fly spray purposes, it was entirely free of the thiocyanate odor usually associated with compounds containing this group.

The β-thiocyanoethyl ethers of the cresols and 1,3,5-xylenol (Table VIII) had very sharp odors. Increasing the carbon content of the thiocyanoalkyl group caused a diminution in this disagreeable odor. The 6-thiocyanohexyl ether of 1,3,5-xylenol was also devoid of this penetrating smell and had an LD50 of 0.93 per cent. In addition to lessening the thiocyanate odor, increasing the thiocyanoalkyl group caused greater solubility of the compounds in refined kerosene.

SUMMARY

1. The toxicity of allyl phenols and the γ-thiocyanopropyl and β-thiocyanoethyl ethers of various phenols to houseflies (*Musca domestica* L.) as

determined by the Peet-Grady method is presented by the formula of their dosage-mortality curves.

2. Allyl phenols were found to possess insecticidal properties. Increasing the number of nuclear allyl groups increased the toxicity of the phenol to flies. The median lethal doses for *o*-allylphenol, *o,o'*-diallylphenol, and *o,o',p*-triallylphenol were 18.5, 10.5, and 2.63 per cent respectively.

3. The γ-thiocyanopropyl and β-thiocyanoethyl ethers of phenols were very toxic to flies and had a very rapid paralyzing effect. Their median lethal doses varied between 0.60 and 1.33 per cent.

4. The γ-thiocyanopropyl ether of 1,3,5-xylenol was found to be an excellent toxicant for use in household fly sprays because of its toxicity (LD_{50} 0.79 per cent), rapid paralyzing effect, and lack of objectionable odor.

ACKNOWLEDGMENT

The authors gratefully acknowledge the help of Albert Hartzell in conducting the biological tests.

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TOXICITY OF PIPERINE SOLUTIONS TO HOUSEFLIES

EDWARD K. HARVILL, ALBERT HARTZELL,
AND JOHN M. ARTHUR

The use of organic compounds to increase the toxicity of household fly sprays containing pyrethrum extracts is of importance as a means of conserving the available supplies of pyrethrum products. The rapid paralyzing action of the pyrethrins is essential for effective fly sprays. This unique knockdown effect which the pyrethrins possess at very low concentrations is difficult to duplicate and until other compounds are found which possess this property and can be used with the same safety the pyrethrins cannot be entirely replaced. Various materials have been used successfully to replace part of the pyrethrins. These substances which usually have little knockdown of their own greatly increase the toxicity of fly sprays. Pyrethrum solutions containing added amounts of N-isobutyl undecylenamide (2, 11) are as effective as solutions containing four times the same amount of pyrethrins. Similarly it was observed that sesame oil (4, 6) markedly increased the effectiveness of the pyrethrins. We have found that the addition of piperine to a pyrethrum solution gives a product that is extremely efficient as a household fly spray. Piperine was found to be more toxic than the pyrethrins but its paralyzing action was too slow to provide the knockdown necessary in fly sprays. Various amides similar to piperine were tested with pyrethrum

METHODS AND MATERIALS

Toxicity tests. The fly sprays were made by dissolving the amides in absolute alcohol and adding a solution of pyrethrum of known concentration. Enough alcohol was used to keep the amide from precipitating when the solutions were made to volume with "Deo-base." The solutions were tested by the large Peet-Grady method (9) and the results were compared with the average of three tests of the Official Test Insecticide (O.T.I.) made at the same time.

Amides. The amides were prepared by refluxing a benzene solution of the acid chloride and an excess of the appropriate amine for one hour. Water was then added and the benzene was evaporated under vacuum. The amide was filtered from the aqueous solution and washed with dilute ammonium hydroxide and hydrochloric acid to remove any unreacted acid and amine. The amide was recrystallized from an organic solvent. Nitrogen values were checked by the Kjeldahl method. The melting points of the amides are listed in Table II.

Acids. Piperic acid was secured from the hydrolysis of piperine with alcoholic potassium hydroxide in yields of 50 per cent. M. p. 216° (7).

3,4-Methylenedioxybenzoic acid was made by the action of 60 per cent potassium hydroxide on an alcoholic solution of piperonal. The yields were almost quantitative. M. p. 226° (3).

Cinnamenylacrylic acid was made by the condensation of cinnamaldehyde, acetic anhydride, and sodium acetate. M. p. 168° (10).

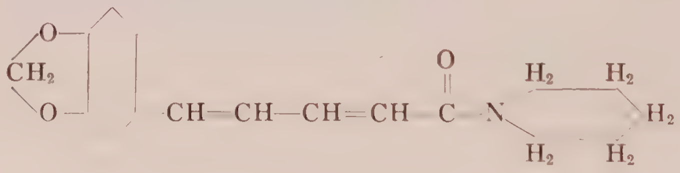
Furylacrylic acid was made by the condensation of furfural, acetic anhydride, and sodium acetate. M. p. 139° to 140° (1).

Amines. 3,4-Methylenedioxybenzylamine was made by the reduction of the oxime of piperonal with sodium amalgam in 66 per cent yield. B. p. 15 mm. 137° to 138° (8).

Cyclohexylamine, aniline, *p*-toluidine, piperidine, and *n*-butylamine are available commercially.

RESULTS AND DISCUSSION

In a study of various plant materials for possible use as insecticides it was observed that the addition of acetone extracts of the dried fruits of black pepper (*Piper nigrum* L.) to pyrethrum sprays caused a great increase in the toxicity of such solutions when tested by the Peet-Grady method. The active constituent in the acetone extracts was found to be the alkaloid piperine, the piperidide of piperic acid.



Piperine

The pure alkaloid, a white crystalline solid melting at 128° to 129°, was available commercially and was used in all the experiments. Acetone extracts of black pepper are unsuited for use in fly sprays since they contain an oil with a very sharp pungent odor. Piperine is almost without odor and causes no nasal irritation. The results of the Peet-Grady tests of piperine are given in Table I.

Solutions of piperine in "Deo-base" containing 10 per cent absolute alcohol gave a knockdown of 85 per cent in ten minutes at a concentration of 0.50 per cent. The toxicity of the alkaloid was determined by the Peet-Grady method by leaving the flies in the chamber overnight. The chamber was ventilated as usual after ten minutes and food was made available to the flies. The next morning the dead flies were counted and those alive were sprayed with pyrethrum and then picked up and counted. This procedure

was necessary since with concentrations of less than 0.50 per cent the knockdown was negligible in ten minutes and the mortality was limited by the knockdown. At a concentration of 0.05 per cent, piperine was superior to the O.T.I. which contains 0.10 per cent of the pyrethrins.

Mixtures of the pyrethrins and piperine were very toxic to houseflies (*Musca domestica* L.). The total concentration of these constituents could be reduced to 0.05 per cent. Such solutions were greatly superior to the O.T.I. A mixture containing 0.20 per cent piperine and 0.10 per cent of the pyrethrins gave a kill equal to the knockdown of 99.8 per cent. The results of Peet-Grady tests of solutions containing varying amounts of piperine and pyrethrum are given in Table I.

TABLE I

TOXICITY OF PIPERINE AND PIPERINE-PYRETHRIN MIXTURES TO HOUSEFLIES

Concn. g. per 100 cc.		Per cent kill 24 hours	Per cent knockdown 10 min.	No. flies	O.T.I. per cent kill
Piperine	Pyrethrins				
0.50	—	81.0	—	429	46.0
0.10	—	75.0	—	421	51.1
0.05	—	72.5	—	341	66.7
0.20	0.10	99.8	99.8	483	59.9
0.10	0.05	99.0	100	473	59.9
0.10	0.03	96.0	100	402	59.9
0.05	0.10	99.3	99.8	419	55.7
0.05	0.05	92.0	100	428	59.9
0.05	0.03	88.3	99.3	705	49.9
0.05	0.02	70.8	99.4	642	49.9
0.05	0.01	45.2	97.1	653	49.9
0.03	0.05	73.9	99.2	392	29.5
0.03	0.03	65.0	99.5	429	29.5
0.03	0.02	64.2	99.9	361	48.7
0.02	0.05	92.0	96.3	588	62.6
0.02	0.03	71.0	100	336	52.0

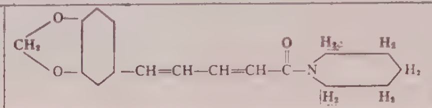
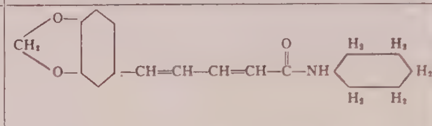
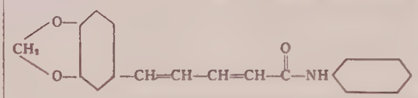
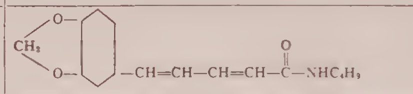
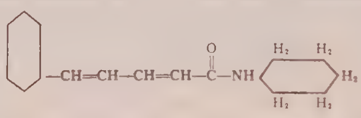
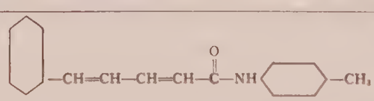
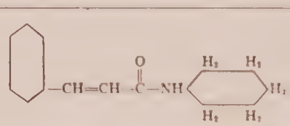
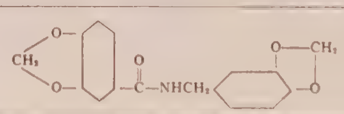
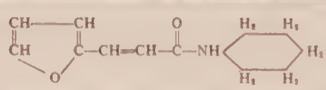
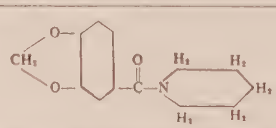
Substituted amides of various acids were synthesized to determine the effect a change in structure would make in the toxicity of the amide-pyrethrum solutions. These amides were tested by the Peet-Grady method, the sprays containing 0.50 per cent of the amide and 0.05 per cent of the pyrethrins. The results of these tests are given in Table II.

Mixtures of the N-substituted piperamides and pyrethrum were very toxic. N-Cyclohexyl and N-butyl piperamide were as effective as piperine. The importance of the methylenedioxy substitution in the phenyl group in piperic acid was made evident by the low mortality of flies with solutions of N-cyclohexyl cinnamenylacrylamide and pyrethrum. Similarly, pyrethrum solutions containing N-cyclohexyl and N-phenyl cinnamides were found to be inactive whereas Gertler, Fales, and Haller (5) found that N-substituted piperonylamides were effective when used with pyrethrum.

Although N-3,4-methylenedioxybenzyl-3,4-methylenedioxybenzamide

TABLE II

TOXICITY OF AMIDE-PYRETHRUM SOLUTIONS TO HOUSEFLIES

Amide used (0.5% amide and 0.05% pyrethrins, except with piperine which was 0.2% amide and 0.1% pyrethrins)		M.P.	Per cent		No. flies	O.T.I. Per cent kill
			Kill	Knock-down		
Piperine		128–129°	99.8	99.8	483	59.9
N-Cyclohexyl piperamide		204–205°	98.5	99.7	922	40.4
N-Phenyl piperamide		203°	58.4	97.7	614	51.3
N-*n*-Butyl piperamide		147°	94.1	99.2	628	51.3
N-Cyclohexyl cinnamenylacrylamide		183–185°	38.2	99.2	620	51.3
N-para-Tolyl cinnamenylacrylamide		186°	39.4	99.1	670	51.3
N-Cyclohexyl cinnamide		176°	29.0	97.4	882	49.4
N-3,4-Methylenedioxybenzyl-3,4-methylenedioxybenzamide		136–137°	58.0	98.5	699	40.2
N-Cyclohexyl furylacrylamide		161°	90.4	99.3	880	49.4
Piperidide of 3,4-methylenedioxybenzoic acid		225°	34.1	98.0	676	40.2

showed definite toxicity with the pyrethrins, the effectiveness of the piperamides appears to be influenced not only by the methylenedioxyphenyl group but also to a lesser degree by the length of the side-chain attached to the phenyl group. A solution containing 0.50 per cent of the piperidide of 3,4-methylenedioxybenzoic acid and 0.05 per cent pyrethrins did not equal the O.T.I. in mortality whereas concentrations of 0.05 per cent of piperine and 0.05 per cent pyrethrins gave a 92.0 per cent kill.

N-Cyclohexyl furylacrylamide was as effective as N-cyclohexyl piperamide.

SUMMARY

1. Piperine, the alkaloid found in the dried fruit of black pepper, is more toxic than pyrethrum to houseflies (*Musca domestica* L.). At concentrations of 0.10 per cent, piperine killed 75.0 per cent and the pyrethrins 51.1 per cent of the flies by the Peet-Grady method.

2. Peet-Grady tests were made by mixing piperine and pyrethrum in various proportions. Fly sprays containing 0.05 per cent piperine and 0.01 per cent pyrethrins were more toxic than sprays containing pyrethrins alone at a concentration of 0.10 per cent.

3. Peet-Grady tests were made of solutions of various substituted amides and pyrethrum. The presence of a methylenedioxyphenyl group increased the effectiveness of the amides. Amides of piperic acid were more effective than the amides of cinnamenylacrylic acid in increasing the toxicity of pyrethrum solutions.

4. Increasing the side-chain attached to the methylenedioxyphenyl group increased the effectiveness of the amide. Piperine was more effective than the piperidide of 3,4-methylenedioxybenzoic acid in increasing the toxicity of pyrethrum solutions.

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A GREENHOUSE METHOD OF EVALUATING FUNGICIDES BY MEANS OF TOMATO FOLIAGE DISEASES[1]

S. E. A. McCallan and R. H. Wellman[2]

There is a need for rapid and precise methods of testing protectant fungicides on the foliage of potted plants in the greenhouse. The procedure should provide an intermediate step between *in vitro* laboratory methods and time-consuming field tests. Little had been done on the development of such a method until the advent of Hamilton and Weaver's (7) apple scab and cedar-apple rust technique. Nielsen (15) has recently successfully adapted this procedure for use with Late Blight on potato foliage and has furnished a detailed description of the technique.

In perfecting a greenhouse technique of evaluating fungicides, advantage should be taken of recent developments in bioassay. Some five years ago a detailed analysis was initiated of the factors causing variation in laboratory spore germination tests of fungicides, and a fairly comprehensive knowledge of this method has been obtained (12, 13, 14, 18). On the basis of this experience and that of the above workers, a method of testing foliage fungicides in the greenhouse has been developed and analyzed, and will be discussed in this paper. Another paper (19) will correlate the results of laboratory and of greenhouse methods of testing fungicides.

REQUIREMENTS FOR A GREENHOUSE METHOD

The requirements of a desirable method of testing fungicides on the foliage of potted plants may be outlined as follows:

Host plant. The host plant should be grown with ease and rapidity, should develop uniformly, be of a fairly open habit of growth suitable for spraying and robust enough to withstand spray pressure; the surface of the leaves should allow adequate wetting by typical fungicides; the plant should not be unduly sensitive to spray injury; also preferably the plant should represent a crop of importance. Slow-growing plants occupy valuable greenhouse space and are frequently subject to attack by other plant diseases and insect pests.

Fungus. The fungus should be readily cultured and produce an abundance of pathogenic spores in a relatively short time. It should be representative of one of the major groups of plant disease fungi.

Disease. The disease should be readily obtained following simple procedures of inoculation and incubation; it should be reproducible; the lesions

[1] A preliminary report on this paper was accepted for presentation before the American Phytopathological Society, New York, N. Y., December, 1942 (10).

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should develop rapidly, be seen with ease, and be suitable for quantitative measurement. The disease should not spread under normal greenhouse conditions. Finally, it is preferable that the disease be of economic importance.

Spraying equipment. The spraying equipment should give reproducible deposits on any given area of the plant foliage; and should cover both upper and lower leaf surfaces uniformly. The equipment should function properly with small quantities of spray, e.g., 100 cc. It should possess adequate adjustments regarding pressure, position, and shape of cone. The sprayer should not clog easily, it must be easily cleaned and maintained. The high pressures of the field equipment cannot be used on potted plants; however, pressure somewhat in excess of that used for laboratory slide tests is probably preferable.

SELECTION OF TOMATO, AND ALTERNARIA BLIGHT, PHYTOPHTHORA BLIGHT, AND SEPTORIA LEAF SPOT DISEASES

The tomato (*Lycopersicon esculentum* Mill.) is one of the few plants meeting all the requirements outlined above, and is a logical choice. Bean plants possess many desirable qualities and studies are in progress with several different diseases. Preliminary comparisons of tomato with potato demonstrated the superiority of the former in many respects. Potato plants do not develop uniformly, at certain seasons dormancy-breaking treatments of the tubers may be necessary, and the plants wilt more rapidly with Phytophthora Blight than do tomatoes. The tomato Bonny Best was chosen from among 15 varieties as being suitable. The details of these varietal tests will be described later.

The three tomato foliage diseases selected for comparative study are all of economic interest, and the causal fungi are representative of some of the most important plant disease fungi. *Alternaria solani* (Ell. & Mart.) Jones & Grout, the cause of the important Early Blight of potato and tomato, may be considered representative of the order Moniliales of the Fungi Imperfecti. Likewise *Septoria lycopersici* Speg. is a member of an economically important genus, of the order Sphaeropsidales of the Fungi Imperfecti. The Phycomycetes are represented by *Phytophthora infestans* (Mont.) de Bary, cause of Late Blight of potato and tomato, outstanding among all plant disease fungi. The leaf mold disease of tomato caused by *Cladosporium fulvum* Cke., of much concern in greenhouses, was not included because of the danger of spread.

SPRAYING OF PLANTS

GENERAL DESCRIPTION

A special room was equipped for the spraying and inoculation of the plants. The equipment may be seen in Figures 1, 2, and 5. The room con-

tains a work bench, sink, hood, turntable, the spraying and inoculation equipment, and two humidity incubation chambers. The plants are brought into the room on hand trucks, placed on a turntable within the hood, and sprayed with the fungicide. After spraying, the plants are removed to the bench or trucks and allowed to dry. If desired, an artificial rain may then be applied. After drying they are again returned to the turntable, inoculated with a spore suspension, and immediately placed in the incubation chambers. After remaining in the incubation chambers for 24 hours the plants are returned to the greenhouse to await the development of disease symptoms.

SPRAYING APPARATUS

The spraying apparatus is illustrated in Figure 1 A. A "paint spray gun"[3] appeared the most desirable for this kind of work and after examining various types a DeVilbiss touch up gun, type CH, was selected. This type is smaller than the usual spray guns and uses less liquid in a given time. A valve regulates the ratio of air to liquid volume, while the spray cone may be adjusted by degrees from circular to a horizontal or vertical ellipse. The gun was mounted in a fixed horizontal position on a galvanized pipe arm, attached to the wall above the work bench. When in use (Fig. 1 B) the arm is held at right angles to the wall; when not in use it is swung against the wall. The air pressure line from the gun is connected by way of a reducing valve to the air pressure system of the building. The fungicide suspension is held in a beaker placed on a rimmed metal plate. This plate is attached to the arm so that it may be moved sidewise or up and down. Also attached to the arm is an electric stirrer.[4] The entire end of the arm, a right angle pipe, to which is fastened the gun, stirrer and beaker plate, may be elevated or lowered according to the height of the plant. Various heights are indicated on the tube and a given position is maintained by a thumb screw.

TURNTABLE

The sprayer when in use is directed toward the hood, inside of which is placed the plant turntable. The hood is equipped with a water nozzle for rinsing and a drain. The turntable is made from a spring winding phonograph and is illustrated in detail in Figure 2. A stainless steel top was fastened permanently above the original turntable. It is fitted with a drop rim to help protect the mechanism which is enclosed in a metal box. The stainless steel turntable is 12 inches in diameter and will carry a single plant. A special compound turntable was made to carry two, three,

[3] The authors are indebted to Drs. Donald E. H. Frear and H. W. Thurston, Jr., of the Pennsylvania State College, State College, Pa., for the initial suggestion of a paint spray gun.

[4] Variable speed electric non-sparking stirrer.

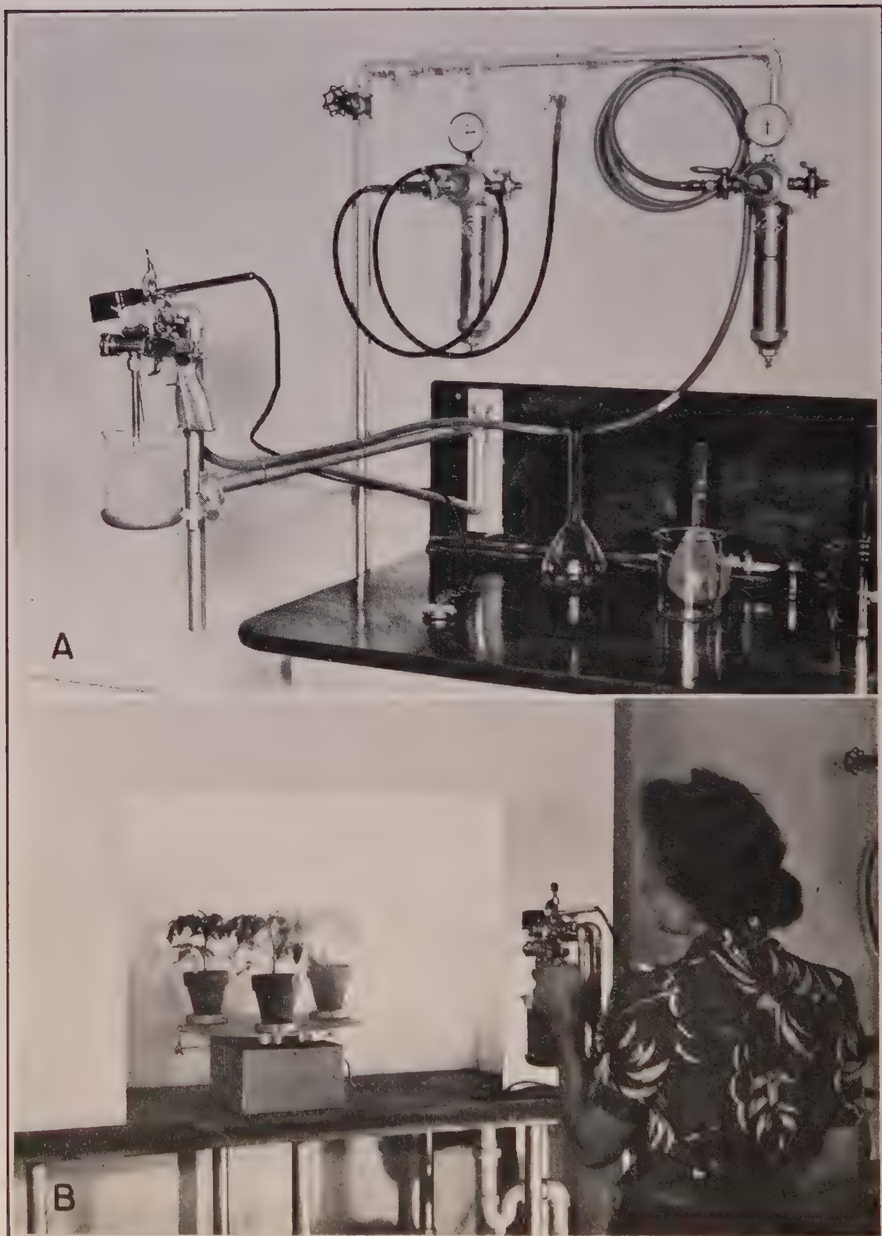

FIGURE 1. A. Spraying systems. Upper left, low pressure for inoculation; upper right, high pressure for applying fungicide. Note swinging arm mounting spray gun, height adjustment, attached stirrer, and rimmed plate holding beaker containing fungicide. B. Spraying tomato plants on compound turntable in hood, with fungicide at 40 lbs. pressure.

or four plants. The turntable is a disc of Transite 18 inches in diameter which can be placed on top of the single turntable. The Transite disc is fitted with six perpendicular pins 1-1/4 inches high. Each of the pins is 7 inches from the center of the main turntable, but they are spaced so that four constitute a square, and one of these plus the remaining two make a triangle, as shown in Figure 2. Four redwood discs were made, 4 inches in

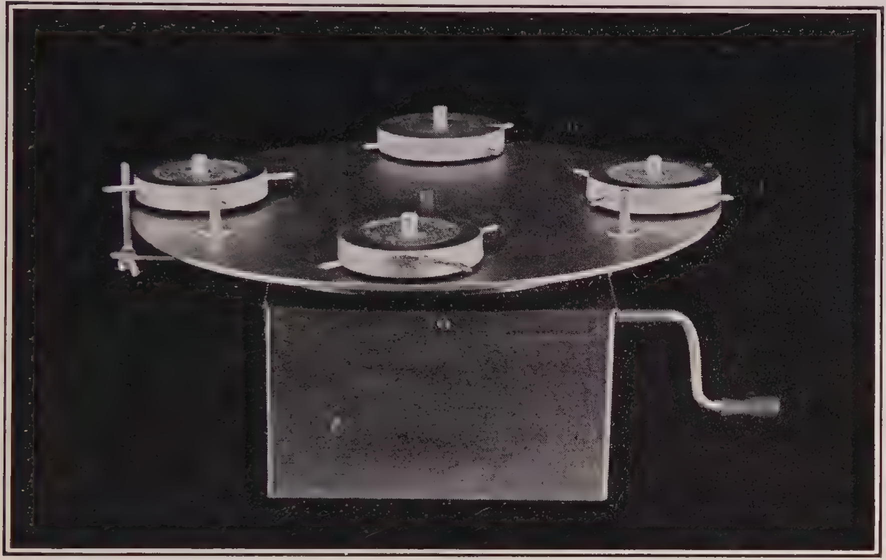

Figure 2. Compound turntable equipped to carry four plants. For two plants remove opposite secondary turntables; for three plants mount secondary turntables on pins at 4, 8, and 12 o'clock positions, see arrangement in Figure 1 B; for one plant entire compound turntable is removed.

diameter and 3/4 inch thick. Four steel pins which project 1 inch are screwed into the side of each disc, 90 degrees apart. The center of each disc is fitted with a capped steel sleeve, which is placed over the perpendicular pins of the main turntable, so that the discs may rotate freely. Two, three, or four discs are used, depending on the number of plants to be sprayed at one time. The discs must be so placed that the turntable is balanced. The potted plant is placed directly on the center of the wooden disc, the capped sleeve, extending upward into the hole in the bottom of the pot, anchors the potted plant. There is mounted on the side of the turntable box an upright rod about 1/8 inch beyond the compound turntable; as the main turntable revolves, the pins of the secondary turntables or wooden disc contact it and each is given 1/4 turn. The speed of the main turntable when loaded with potted plants is regulated to approximately 20 revolutions per minute. The center of the main turntable is 50

inches from the nozzle of the spray gun. Ordinarily for a single or for several plants of spreading habit on the turntable the horizontal spray ellipse adjustment of the spray gun is used. In the case of tall single plants the vertical ellipse adjustment is employed. Actual spraying operations showed that, due to the large volume of spray, it was necessary to leave hood draft on full.

CALIBRATION OF SPRAY GUN AND MEASUREMENT OF TOMATO PLANT

The spray gun was calibrated for delivery, and measurements made of the leaf area of the tomato plant and of the spray deposit for the conditions described on the preceding pages.

TABLE I

SPRAY DELIVERED BY DEVILBISS CH GUN AT DIFFERENT PRESSURES AND FLUID VALVE SETTINGS. CUBIC CENTIMETERS PER 10 SECONDS OF SPRAYING TIME. MEAN OF 5 DETERMINATIONS

Pounds pressure	Turns on fluid valve						
	0.5	1.5	2.5	3.5	4.5	5.5	6.5
10	1.5	9.6	16.6	21.6	22.8	22.8	23.0
20	2.6	15.4	25.8	31.2	33.6	33.7	34.0
30	3.1	19.4	31.2	39.8	42.2	42.6	42.8
40	4.1	23.2	38.0	50.6	52.8	53.8	53.8
50	5.2	25.2	42.4	55.6	59.6	61.0	61.4
60	5.6	26.6	43.8	61.0	63.6	64.4	65.8

Delivery by gun. The amount of spray material taken up in 10 seconds of spraying time at various air pressures and fluid valve settings, for this particular gun, is given in Table I. For example, at 40 lbs. pressure and 2-1/2 turns open of the fluid valve, 38 cc. of spray will be delivered in 10 seconds.

Measurement of tomato plant. When it was decided to use the tomato variety Bonny Best as a test plant, it became desirable to determine areas of the compound leaves in different positions and at different plant ages or sizes. Due to the variability in light conditions and daytime temperatures in the greenhouse and hence of growth it appeared best to specify the extent of plant growth by plant height rather than by age. The plants are grown in 4-inch pots and the height taken at the crown to the nearest half inch. Leaf position was designated numerically beginning at the first or lowest compound leaf. The leaf areas of three comparable leaves, each of course from a different plant, were measured by means of a planimeter. The leaf was held between a sheet of thin transparent glass and a sheet of ground glass above a light source, and its outline traced with pencil on onion skin paper. The area of the tracing was then determined with the planimeter. In the results given in Figure 3, the area indicated is for both

leaf surfaces. The family of curves is very well defined considering only three leaves were measured at each point. It will be observed that for small plants, i.e., 4 inches tall, the third or fourth leaf is largest, while with age successive leaves become the largest. Note that with 6-inch plants leaves Nos. 4, 5, and 6 are the largest.

Measurement of Spray Deposit on Leaf

The amount of spray deposited on the leaves was determined by spraying with a Standard Laboratory Bordeaux (1) suspension containing 0.05

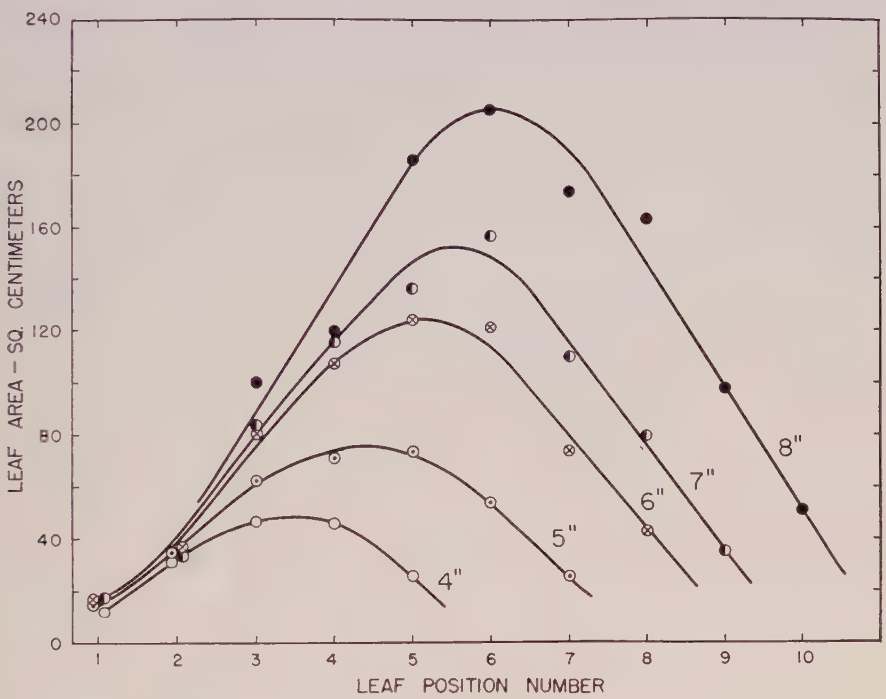

Figure 3. Calibration curves for Bonny Best tomato plants showing relation of leaf area (both surfaces) to leaf position for plant heights of 4, 5, 6, 7, and 8 inches respectively. Each point mean of three plants.

per cent copper. Individual leaves were washed in 100 cc. of 2 per cent sulphuric acid for a few minutes. The leaves were then rinsed in fresh water and their area measured by a planimeter as above. The copper in solution was determined by the sodium diethyl dithiocarbamate test (4). Knowing the amount of copper obtained from a given leaf and the area of the leaf, the amount of spray deposited per unit area of leaf can be derived.

Tests were first made to determine the height of the spray gun relative to the plant height. Three plants each were sprayed with the gun at crown

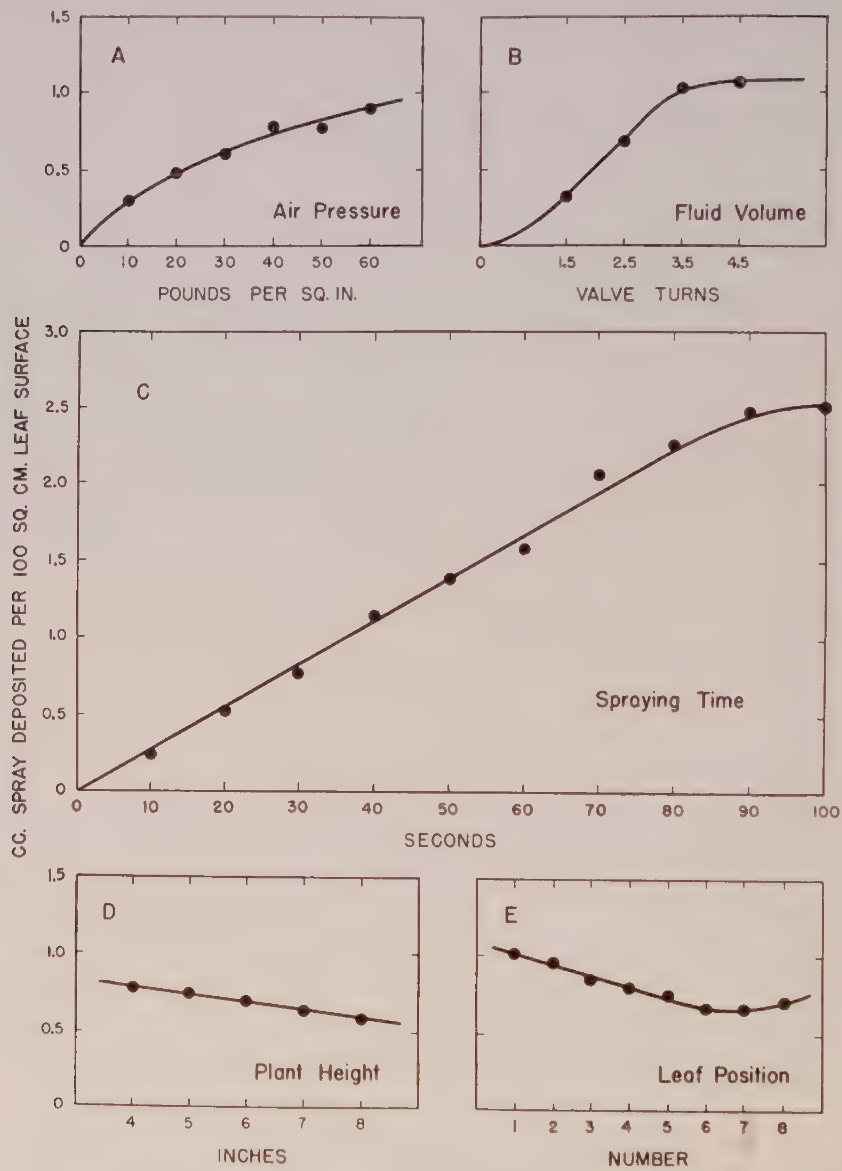

FIGURE 4. Calibration curves for deposition of spray on tomato leaves var. Bonny Best, 3 plant compound turntable. A. Air pressure; B. fluid valve setting; C. spraying time; D. plant height; and E. leaf position. Factors held constant while others varied: air pressure, 40 lbs.; fluid valve, 2.5 turns; spraying time, 30 seconds; plant height, 6 inches; and leaf position, 4, 5, and 6. Each point mean of 9 determinations, i.e., 3 leaves from each of 3 different plants, except D and E where 1 leaf from each of 3 plants.

height and 1, 2, and 3 inches below. An analysis of variance on deposit from the three replicate plants at the four levels indicated a barely significant difference. Two inches below the crown level of the plant was selected as the standard height for the gun position.

The factors air pressure, fluid valve settings, time of spraying, plant height, and leaf position were determined several times for both single and three plant compound turntables. The results for the most comprehensive test on a three plant turntable are recorded in Figure 4. Each point is the mean of three leaves, Nos. 4, 5, and 6, from three different 6-inch plants, except in D and E where there is only one leaf from each of three plants. The deposit curves for air pressure and fluid valve settings (Fig. 4 A and B) agree in shape with those of the corresponding delivery curves, the data for which are given in Table I. The amount of deposit is directly proportional to spraying time (Fig. 4 C) up to about 80 seconds when run-off commences. Run-off time will of course vary with the physical nature of the spray. However, under these conditions it would be possible to apply four different doses from the same stock suspensions, with a dose ratio of 2, by spraying for 10, 20, 40, and 80 seconds. The taller plants receive less spray per unit area than the shorter ones (Fig. 4 D) and the older or lower leaves receive more than the younger leaves, except for the very youngest where the deposit curve tends upward (Fig. 4 E). Similar results were observed with 4, 5, 7, and 8-inch plants.

A comparison was made of the amount of deposition on tomato plants on single and 2, 3, and 4 plant turntables. The single plant was placed in the center of the turntable while the other plants were inserted on the compound turntables as described above. From two to four replicate tests were made on 6-inch plants for each turntable and deposition determined for leaves Nos. 3, 4, 5, and 6. All other variables were held constant. The mean amount deposited for 1, 2, 3, and 4 plant turntables was respectively 1.005, 0.667, 0.677, and 0.608 cc. per 100 sq. centimeters. No significant difference could be shown for the amount deposited on two or three plant turntables. However, the deposition for the single plant turntable is 50 per cent greater, and that for the four plant turntable is 10 per cent less.

As a result of these tests it was decided to standardize at 40 lbs. pressure, 2-1/2 fluid valve turns and 30 seconds spraying time. Under these conditions approximately 120 cc. of spray would be required for one turntable of three plants. In the case of a 6-inch plant, leaves Nos. 4, 5, and 6 would receive approximately 0.75 cc. of spray deposit per 100 sq. cm. of leaf area. Actual determinations of relative amount of deposit present on lower and upper surfaces were not made. Visual observations of heavy doses indicated that the amounts were about equal, also the spread on a single leaf was reasonably uniform. The combination of air pressure, compound turntable, and air currents produced by hood draft and gravity

tend to result in a uniform coverage and deposit for a given set of conditions.

TESTS WITH ARTIFICIAL RAIN

If desired, sprayed and dried plants may be placed on the compound turntable in the hood and subjected to a laboratory rain. Tap water under its own pressure is sprayed upwards at a 45° angle through a rose type nozzle held above and to the side of the plants. The rain thus describes an arc and hits the plants on the far side of the turntable. The nozzle is of aluminum and was originally designed for sprinkling clothes. Under these conditions the plants will have received an inch of rain in approximately 5 minutes. They must then be set aside to dry. It is planned to develop a machine to handle more plants and longer exposures for this rain resistance test.

CULTURE OF FUNGI

In order to obtain adequate spore production it is necessary to culture the three fungi under different conditions.

Alternaria solani. Few strains of *A. solani* will both produce spores in culture and exhibit pathogenicity. After examining a number from different sources a satisfactory one was obtained from Orono, Maine.[5] The organism is grown on Petri dish cultures of potato dextrose agar at room temperature. In about two weeks the organism covers the dish and a very scant production of conidia has occurred. The surface of the agar is then scraped with a straight edge scalpel to remove all superficial mycelium, according to the method of Kunkel (8). The covers are removed and several dishes are placed right side up in moist chambers. The moist chamber covers are held ajar about 1/8 inch and the whole placed on a sunny window sill, which is essentially the method of Rands (16). In about three days, depending on the weather, abundant spores are produced. The number of spores gradually diminishes with age; dishes from three to ten days after scraping may yield several million spores. The spores are removed from the Petri dishes by means of a rubber policeman, filtered through cheesecloth, counted, and the necessary dilution made for the plant inoculation.

Phytophthora infestans. A virulent strain of *P. infestans* was obtained and cultured on slices of potato tuber, in general according to the methods developed by Crosier (5), Nielsen (15), and associates.[6] Various modifications were tried and the following procedure was adopted. Whole tubers

[5] Isolate No. 52, Maine Agricultural Experiment Station, Orono, Maine, kindly furnished through the courtesy of Dr. Reiner Bonde.

[6] The authors are indebted to Dr. Phares Decker for furnishing this culture, and wish to express their appreciation to Drs. L. W. Nielsen, and J. B. Skaptason, all formerly of the Dept. of Plant Pathology, Cornell University, Ithaca, New York, for suggestions regarding the culturing of this organism and its use as a fungicide test organism on potato.

are inoculated by atomizing the sprouting eyes with a suspension of motile swarmspores. Several tubers are held in a moist chamber at 21° C. for one week. The tubers are then sliced about 1/4 inch thick and stacked loosely on a glass slide rack above moist filter paper in a moist chamber. In several days there is an abundant production of sporangia; these are removed with a camel's hair brush to distilled water containing a little bentonite (5) and held at 10° C. for 6 hours when there is a maximum production of swarmspores suitable for inoculation. A spore count is made by means of a Fuchs-Rosenthal counting cell, and the spores are diluted as required with distilled water at 10° C. The tomato plants are inoculated immediately at room temperature. The question of time for swarmspore production and spore concentration will be discussed further under greenhouse results. Six moist chambers, each containing about 8 slices of potato, will ordinarily furnish enough swarmspores to infect 10 dozen tomato plants. Several crops of sporangia may be grown on the slices provided they do not become too rapidly decayed with secondary organisms.

Septoria lycopersici. This species[7] is cultured readily on potato dextrose agar slants at 21° C., by making a dense planting over the entire surface of the slant. In several weeks numerous pycnidia are formed throughout the agar which is partially ground up with a mortar and pestle in order to liberate the conidia. The spore suspension is filtered, counted, and adjusted to the necessary dilution.

INOCULATION AND INCUBATION OF PLANTS

INOCULATION

Since the fungicide spray gun is not suited for spore inoculations due to the relatively large volumes and high pressure involved, an atomizer has been used. Preliminary tests with various types of atomizers indicated that the DeVilbiss No. 150 is very satisfactory. This is a concentric type with the air tube fitting within the water tube. The spores travel a straight path and the whole apparatus may be readily taken apart for cleaning. In order to reduce wear on the adjustment valve the atomizer was attached to a second reducing valve as illustrated in Figure 1 A.

It has not proved feasible to design a stationary apparatus to hold the spore atomizer; consequently the thoroughness of inoculation is dependent to a degree on the skill of the operator. The spore suspension, prepared as above and at the desired concentrations to be discussed later, is placed in a 7-ounce bottle that is screwed to the atomizer which in turn is attached by means of a cut-off valve[8] to the air hose and reducing valve. The oper-

[7] Obtained through the courtesy of Dr. S. B. Locke, University of Arkansas, Fayetteville, Ark., who suggested the method of culturing.

[8] DeVilbiss No. 631.

ator holds the bottle and cut-off in hand and while shaking the bottle to keep the spores in suspension, moves the apparatus slowly up and down so that the nozzle describes an arc of a circle whose radius is approximately 18 inches from a point 2 inches below the crown of the nearest plant. The arc extends about 45 degrees so that the spores are atomized onto both upper and lower surfaces of the leaf. A stiff wire curved to the necessary arc may be attached to the hood to serve as a guide. The hood damper is kept closed during inoculation and is opened only at intervals to prevent a possible accumulation of spores.

Calibration of spore atomizer. The amount of spore suspension delivered by the atomizer at pressures of 5, 10, 15, 20, 25, 30, and 35 pounds was determined to be respectively 14.0, 16.8, 17.8, 18.7, 19.8, 20.3, and 20.5 cc. per 30 seconds of atomizing time. The change in amount delivered thus rapidly lessens with increasing pressures. A pressure of 20 lbs. was selected as a suitable standard. Three 6-inch tomato plants were placed on the turntable and atomized with a Bordeaux suspension under the above conditions at 20 lbs. pressure for 20 seconds. A second set was atomized for 30 seconds and a third set for 40 seconds. A copper analysis was made as in the calibration of the spray deposit and the amount of liquid deposited was determined per unit area of leaves Nos. 3, 4, 5, 6, and 7. An analysis of variance on the deposits indicated no significant difference for the various leaf positions. The amount of deposit was proportional to the time of atomizing. An atomizing time of 30 seconds was adopted as standard. Three plants each of 4, 6, and 8-inch heights were likewise atomized and the deposit determined. The smaller plants received more deposit and the larger plants less, as was the case with the spray gun. These differences, although significant, were small, the 6-inch plants receiving approximately 12 per cent more than the 8-inch plants and 12 per cent less than the 4-inch plants. As above, no significant trend in leaf position could be shown. This second test on 6-inch plants was performed one month later, by the same operator. The calculated deposition of liquid per 100 sq. cm. of leaf surface at 20 lbs. pressure, 30 seconds of atomizing time, 6-inch plants, and for the mean of leaves Nos. 3, 4, 5, 6, and 7, was 0.162 cc. for the first test and 0.180 cc. for the second. This agreement demonstrates that the hand atomization can give reasonably reproducible results. Under the standard conditions outlined above, a spore suspension concentration of 10,000 spores per cc. would be deposited at a rate of approximately 17 spores per sq. cm. of leaf area, or 34 spores for a sq. cm. of leaf tissue counting both upper and lower surfaces.

INCUBATION

Two glass and wood incubation chambers were built measuring 5 feet wide, 5 feet high, and 2-1/2 feet deep. These are set on pipe legs and are

equipped with a hinged door in the front center, a metal bottom, drain, and removable wooden shelf as illustrated in Figure 5. At the upper side of each chamber there is attached a water valve and an air valve for a Carrier atomizing jet nozzle. In order to protect the plants from larger droplets from the atomizer nozzle, a plate glass shelf was inserted 3 inches below the nozzle. There is from 3 to 4 inches clearance around both wooden and plate glass shelves so as not to interfere with air circulation.

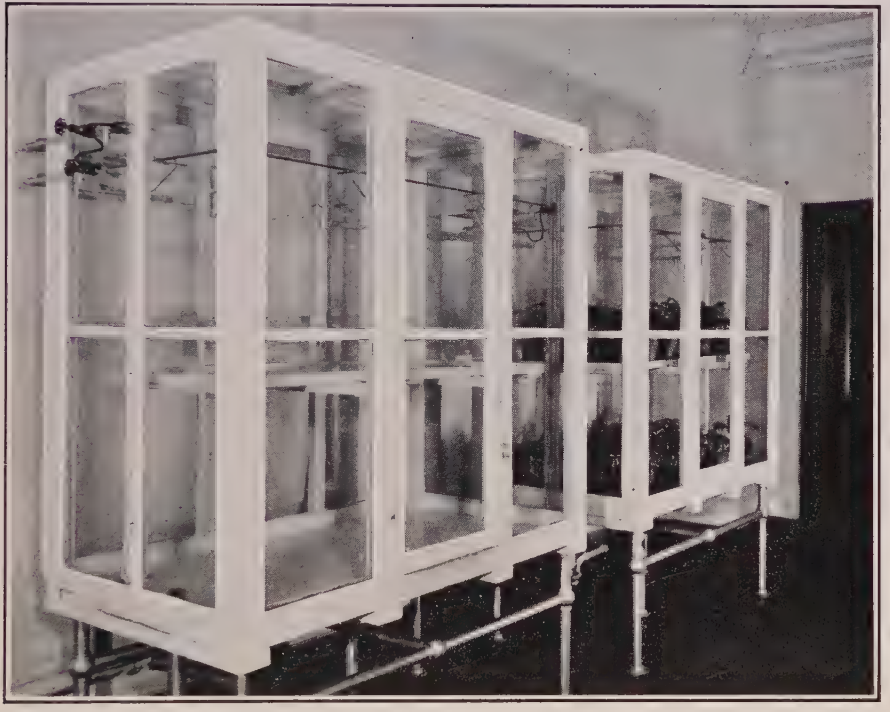

Figure 5. Humidity infection chambers. On upper side of each, Carrier atomizing jet nozzle with water line on top, air line on bottom; pipe on side catches drip. Note protecting plate glass shelf.

When the valves are turned on, a relative humidity of 100 per cent is reached in about 10 minutes. While the plants are being inoculated the valves are turned on, and immediately upon inoculation each plant is placed in the chamber. Each chamber with shelf will accommodate without crowding 60 6-inch tomato plants in 4-inch pots. The humidifying apparatus is run only during inoculation and 1/2 hour after, for 1/2 hour about 8 hours later, and again 16 hours after inoculation. The plants are removed to the greenhouse after 24 hours in the chamber. Usually the plants are inoculated in the late afternoon so that they will be returned

to the greenhouse at a corresponding time and will not be subjected immediately to full sunlight.

Detailed tests have not been made on the effects of different rates of humidifying or relative humidities. The plants remain wet during the 8-hour periods with no humidifying. Limited tests indicated no difference in disease following a 48-hour exposure in the chamber as compared to the standard 24-hour period. The chambers are held at room temperature, approximately an extreme range of 60° to 85° F., though during a single test the range seldom exceeds 5°. During humidifying the temperature within the chambers will drop two or three degrees. These conditions are not necessarily the optimum for any of the three diseases, for the objective has been to arrive at a convenient though perhaps arbitrary set of conditions which result in a satisfactory and reproducible amount of disease in the plants.

FACTORS INFLUENCING THE DISEASE COUNT IN CONTROL PLANTS

A number of factors have been studied which were found to affect the number of disease lesions on the unsprayed plants. These must be examined separately from the effects of the fungicide. The known factors are time of appearance of lesions, variety of tomato, size of plant, leaf position, spore age, spore concentration, and operator errors. In addition to these factors there are apparently others which result in one disease giving more consistent readings than another.

TIME OF APPEARANCE OF LESIONS

The time of appearance of disease lesions cannot be predicted with certainty. This is due, probably in the main, to variation in the susceptibility of the different lots of tomato plants, which in turn is due to variation in the weather, soil, and rate of growth.

Early Blight. Lesions of Early Blight may be expected in from 40 to 60 hours after inoculation. The lesions first appear as small water-soaked pin points, best seen from the under surface by reflected light. Usually they develop very slowly, becoming brown and somewhat angular. They seldom exceed 2 mm. in diameter; however, an unusually severe infection may cause a blighting of the leaves. The lesions are illustrated in Figure 6 A. Fresh lesions may continue to appear over a period of several days though the ratio of lesions in treated plants to those in checks will not change materially as shown in Figure 7. Thus the lesion count may be taken any time over a period of several days. The disease does not spread under greenhouse conditions.

Late Blight. Late Blight lesions usually do not appear before 60 hours after inoculation. They are first seen as dark indistinct spots with fimbriate margins, which rapidly enlarge and coalesce so that in severe cases the

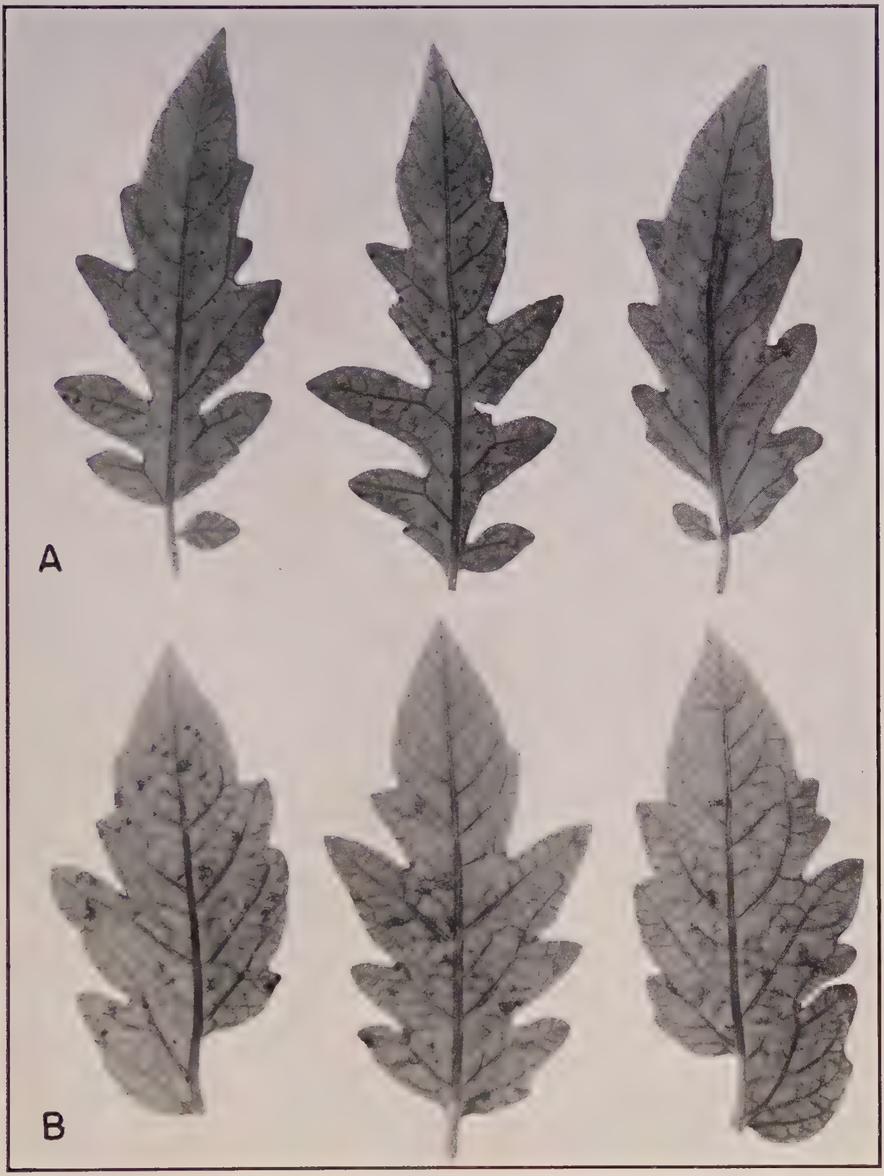

Figure 6. Lesions on tomato leaflets var. Bonny Best. A. Early Blight (*Alternaria solani*). Progressive development of lesions from left to right hand leaves. Note light sunken areas around initial lesion in later development. Number of lesions from left to right, respectively, 33, 80, and 45. B. Late Blight (*Phytophthora infestans*). Lesions at correct stage for counting, before coalescing. Number of lesions, left to right, 53, 25, and 27.

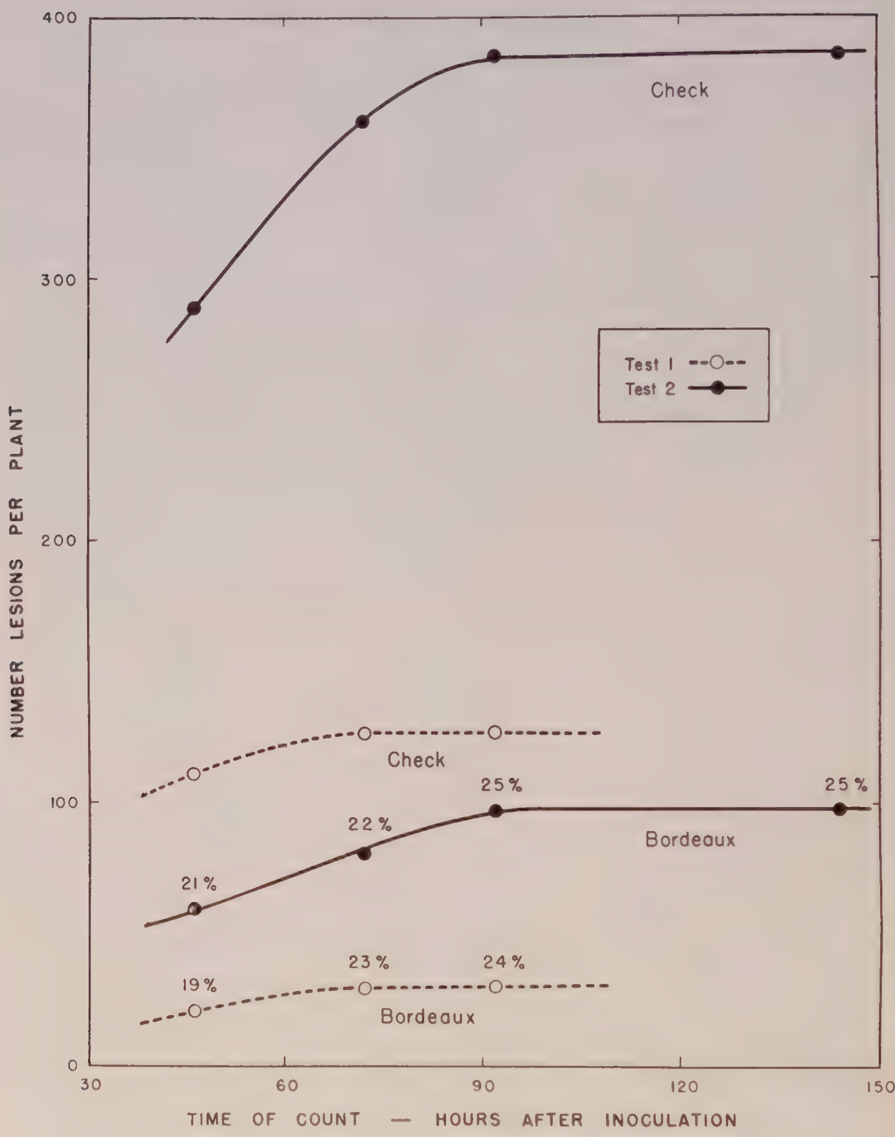

FIGURE 7. Relation of time of counting Early Blight lesions to total number of lesions per plant of 3 leaves, and the difference in number of lesions from 2 tests. All time and test differences tend to be eliminated when the Bordeaux treatments are expressed as per cent of check as indicated above.

plant may be entirely wilted within 24 hours of the first appearance of lesions. Hence it is essential to count the lesions within this 24-hour period. The lesions are illustrated in Figure 6 B. This disease also does not spread in the greenhouse.

Septoria Leaf Spot. The Septoria Leaf Spot disease develops very slowly, lesions not appearing until about 10 days after inoculation. The spots are fairly large, 1 to 2 mm., circular and distinct; in a few days the typical pycnidia may be seen. Unfortunately the disease spreads under greenhouse conditions and if the count is delayed, secondary lesions will have developed.

TABLE II

Varietal Susceptibility to Three Tomato Diseases. Mean Number of Disease Lesions per Three Compound Leaves

Variety	Late Blight	Septoria Leaf Spot	Early Blight
Ponderosa	222	195	
The Orange	206	171	114
Bonny Best	172	187	110
Marglobe	138	281	—
Rutgers	118	163	214
Stone	115	273	—
John Baer	111	128	—
Beefheart	—	—	104
Yellow Beefheart	58	329	—
Dwarf Champion	44	559	28
The Fruit	38	107	—
Beefsteak	35	79	—
Yellow Cherry	34	78	52
Crimson Cushion	32	164	41
Comet	17	71	9
Ratio for significant difference	3.5	2.6	

TOMATO VARIETIES

In the early stages of developing this method it became necessary to standardize on a suitable variety. Accordingly, preliminary tests were made on some 15 different varieties, observations being made on the relative susceptibility and other responses set forth at the beginning of this paper. The varieties tested represented a wide range in growth habit. Two tests with three plants each were made on both Septoria Leaf Spot and Late Blight and one small test on Early Blight. The results on the mean number of lesions per three compound leaves are given in Table II. It will be noted that there is a significant difference in varietal susceptibility. Two tests were also made on the per cent disease control of Late Blight with different doses of Bordeaux mixture; no significant difference could be shown between varieties. These tests, although preliminary in nature,

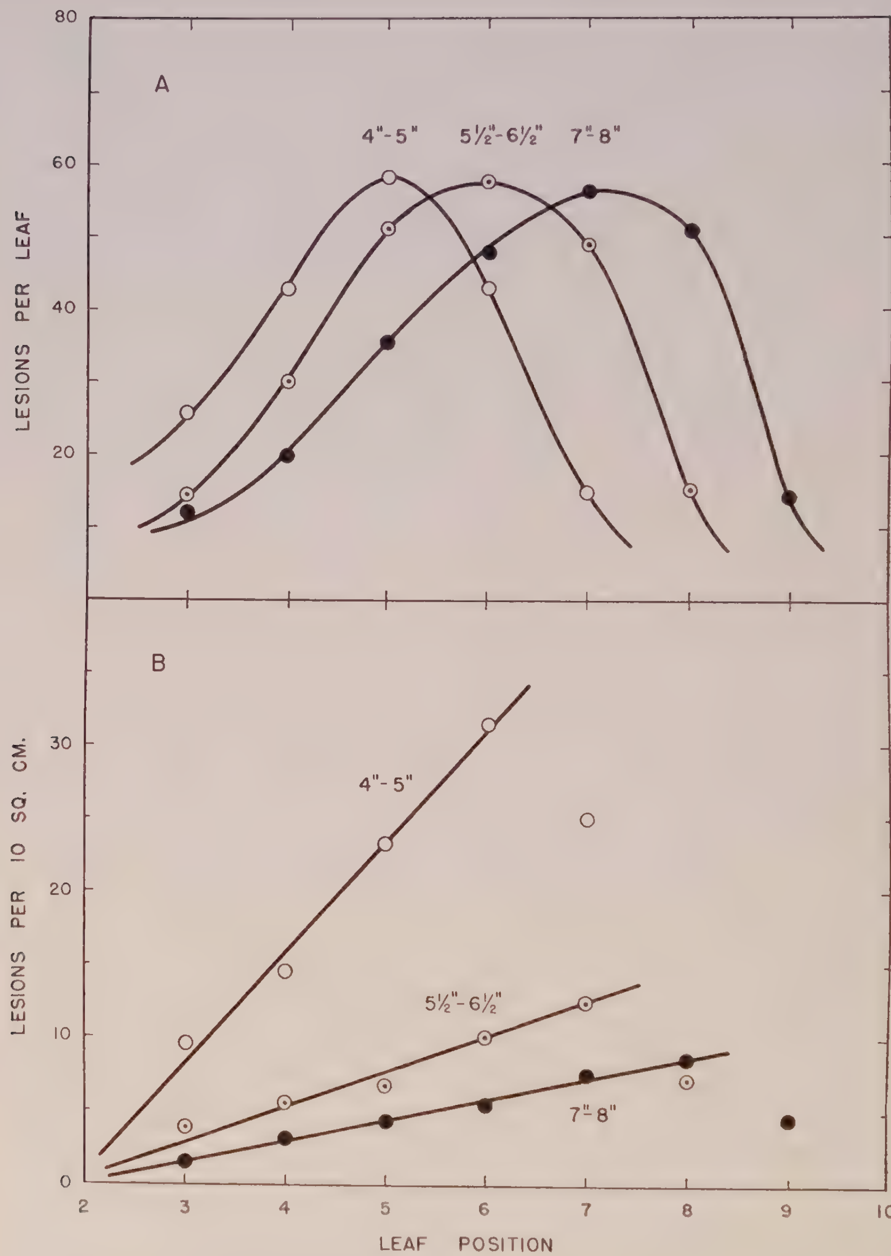

FIGURE 8. Relation of leaf position and plant size to number of Early Blight lesions. A. On basis of lesions per leaf. B. On basis of lesions per unit area of leaf. Each point mean of 3 tests with 2 plants each.

did allow a choice to be made, and on the basis of susceptibility, distinctiveness of disease lesions, growth habit, robustness, and commercial importance, the variety Bonny Best was selected.

SIZE OF PLANT AND LEAF POSITION

A number of tests were made with Early and Late Blights regarding the effect of plant size and leaf position on the number of lesions. The mean results from four experiments with two plants each with Early Blight are shown in Figure 8; generally similar results held for Late Blight. The plants are grouped into three height classes, 4 to 5, 5-1/2 to 6-1/2, and 7 to 8 inches. Since the same four experiments did not always run through all three classes, exact comparisons between the classes cannot be made. It will be seen that within each class there is a distinct difference in distribution of lesions per leaf with a well defined maximum at respectively the 5th, 6th, and 7th leaf. However, if the lesions are expressed on a per unit area basis, the relationship was linear with the youngest leaves (up to a certain point) showing the most spots, and consistent with this the youngest plants having the most lesions. Further tests would be necessary to determine the significance of the apparent drop in number of lesions for the very youngest leaf in each class.

These results show that for a given test the plants must be uniform in size and leaves in similar position should be examined when counting the lesions. It is most efficient to count the leaves with the greatest number of lesions, and three leaves appear to be an adequate number, provided they total 200 or more lesions. The standard procedure has been adopted of recording the lesions on five or so of the leaves of the check plants, determining which three adjacent leaves average the greatest number of lesions. Then in the case of all treated plants the number of lesions is recorded for the three equivalent position leaves. Usually, as seen in Figure 8, this will be leaves Nos. 4, 5, and 6 for 4 to 5-inch plants, 5, 6, and 7 for 5-1/2 to 6-1/2-inch plants, and 6, 7, and 8 for 7 to 8-inch plants. Six-inch plants are preferred where possible.

SPORE AGE

Since the age of the spores markedly affects their germination on glass slides (6), it is to be expected that a similar result might hold for infection. This relation and especially the optimum age for maximum infection was considered of interest.

Early Blight. Tests to date indicate that spores of *Alternaria solani*, obtained from 3 to 6 days after scraping the cultures, produce the greatest number of lesions on tomato leaves. The infective power of the spores in general gradually decreases with age and in 12 days is less than half that at the optimum age. Spore yields vary; as high as several million per Petri

dish may be produced. The factors influencing spore production and infection by *A. solani* are being studied further.

Late Blight. The temperature and time relations for direct and indirect germination of *Phytophthora infestans* are well known (5). Under normal conditions, potato leaves are considered to be infected only by swarmspores, whose optimum temperature for germination is 12° C. (5). Since a temperature of 10° C. was available for obtaining swarmspore production, a determination was made of the length of time to reach maximum swarmspore production under our conditions. Secondly, a study was made of the relative ability of motile, stationary, and germinated swarmspores to infect tomato foliage.

A first crop of sporangia was obtained from potato tubers, six days after slicing. These were suspended in distilled water at 10° C. and at logarithmic time intervals aliquots were taken and examined at 10° C. for type of germination. The time ratio was $\sqrt{2}$, exclusive of 2 vacancies, that is, 0.75, 1.5, 2.125, 3, 4.25, 6, 8.5, 12, 17, 24, 34, 48, and 96 hours after suspending the sporangia in 10° C. distilled water. At each time interval four samples were taken, a total count made in a Fuchs-Rosenthal cell of the spores in 1 cubic mm. for each sample. Records were made of indirectly germinated and ungerminated sporangia, motile swarmspores, stationary swarmspores, and germinated swarmspores. The total sporangia count at each time was of course practically constant, namely approximately 48,000 per cc. As soon as each count was made, an aliquot of the suspension was diluted 1 to 8 and three 6-inch tomato plants inoculated. The results of this test are summarized in Figure 9, where the various swarmspore populations are plotted against time on a logarithmic scale. Since the spore inoculum was diluted 1 to 8, the actual number of swarmspore types observed has also been divided by 8 in order to make the lesions and swarmspore numbers comparable. A repetition of the test gave essentially the same results. In Figure 9 a calculated normal distribution curve (the solid line) was obtained for the motile swarmspore data and found to fit within the limits of error. Thus it may be stated that the population of motile swarmspores of *Phytophthora infestans* is normally distributed against the logarithm of time. The mean or time at which the maximum number of swarmspores are present is 6 hours. As time passes, more swarmspores become stationary so that at 24 hours these are in the majority, while at 96 hours no motile swarmspores are present and the majority have germinated. The distribution of Late Blight lesions against time will be seen to agree closely with that of the motile swarmspores. Hence it may be concluded that only motile swarmspores can infect tomato foliage, and the number of infections is proportional to the number of motile swarmspores.

Septoria Leaf Spot. One test with spores of *Septoria lycopersici* from

2-, 3-, and 4-week-old slants showed no significant difference in numbers of leaf spot lesions.

SPORE CONCENTRATION

Considerable data have been accumulated on the relation between spore concentration and number of lesions for all three diseases. It has been observed from test to test that even though the spore concentration and

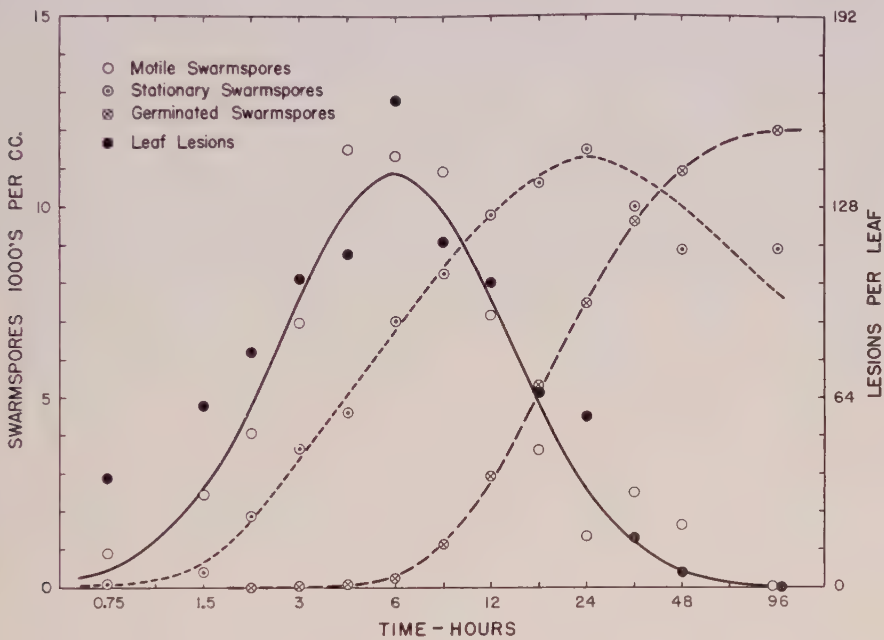

FIGURE 9. Relation of length of time sporangia of *Phytophthora infestans* were immersed in distilled water at 10° C. to the population of motile, stationary, and germinated swarmspores. Also the relative infectivity of the population as shown by Late Blight leaf lesions. Note that number of lesions is directly proportional to the number of motile swarmspores.

age are held constant there are variations in the total number of lesions. Presumably this is due mainly to fluctuations in the sensitivity of the tomato plants. However, within a test the effect of spore concentration is clear cut. Typical results based on one test comprising three 6-inch plants, and three leaves per plant, Nos. 4, 5, and 6, for each of the three diseases are shown graphically in Figure 10. The wide range of values covered necessitated the logarithmic scale of plotting. It will be observed that in all cases there is a constant ratio between the number of lesions and the number of spores. Nielsen (15) found a similar relation for Late Blight of potato. The percentage infection is highest with *Phytophthora* spores and lowest with *Septoria*. Approximately 200 lesions on three ma-

ture leaves of a plant may be expected to result from an initial spore inoculum of 5,000 spores per cc. for *Phytophthora*, 25,000 for *Alternaria*, and 200,000 for *Septoria*. These concentrations have been adopted as more or less standard.

The per cent of spores actually infecting the leaf may be calculated. It has been shown that under standard conditions of atomizing, 17 spores will be deposited per sq. cm. of leaf area from a suspension containing

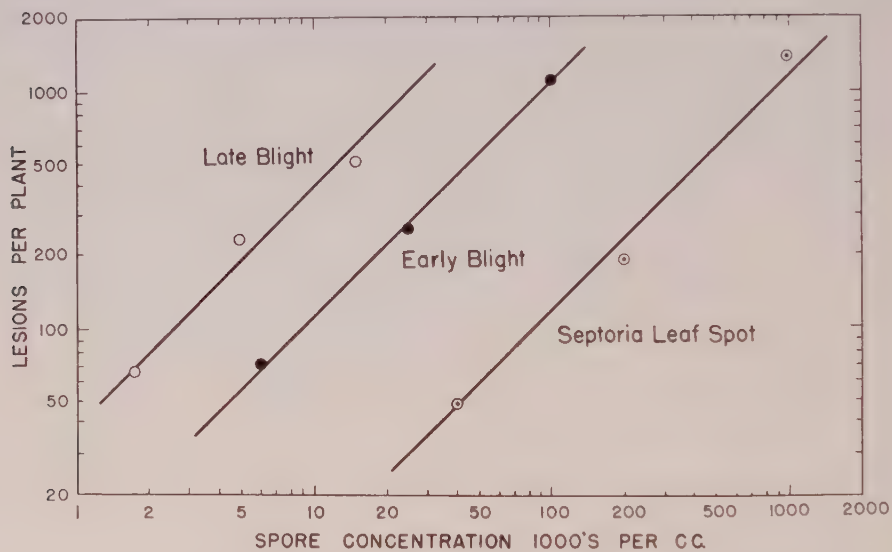

FIGURE 10. Relation of spore concentration to number of lesions of the three tomato diseases. Each point mean of 3 plants with 3 leaves each.

10,000 per cc. The approximate total leaf area for leaves Nos. 4, 5, and 6 from 6-inch plants may be seen from Figure 3 to be 360 sq. cm. Therefore, the total number of spores deposited on the three leaves from a suspension of 10,000 per cc. is 17×360 or 6120, or 18,360 from a suspension of 30,000 spores per cc. From Figure 10 it will be seen that 30,000 spores per cc., or 18,360 actually on the leaves, will produce respectively 1200, 320, and 35 lesions of Late Blight, Early Blight, and Septoria Leaf Spot. Hence, the approximate per cent infection by spores of *Phytophthora infestans* is $(1200/18{,}360) \times 100$ or 6.5; by *Alternaria solani* is 1.7; and by *Septoria lycopersici* is 0.2.

OPERATOR COUNT VARIATION

In extreme cases where the disease lesions are very small, or where they have coalesced, it may become a matter of opinion as to what constitutes a single lesion. Under these circumstances errors may be introduced by the

individual operators. The operator may consistently obtain too high or too low counts, or he may be erratic either within or between tests. Six experienced operators participated in two tests on counting Early Blight lesions. Each operator independently counted the lesions on the same leaves for the same eight plants. The total counts obtained by each operator in the first test are given in Table III. An analysis of variance on the individual counts showed a highly significant difference for the operators. A week later the test was repeated, the operators in the meantime having seen the results of the first test. The total counts for the second test are also given in Table III and again there was a highly significant operator

TABLE III

TOTAL EARLY BLIGHT LESIONS COUNTED BY DIFFERENT OPERATORS

First test		Second test	
Operator	Total lesions	Operator	Total lesions
C	1427	T	1941
M	1292	W	1710
T	1291	A	1510
S	1252	C	1505
A	1248	S	1447
W	1101	M	1374

difference. It will be noticed the order of the operators is markedly changed in most cases, those counting high the first time counted low the second, and vice versa. This apparently was due to an attempt to correct for their earlier count, but resulted in an over-correction. It was also observed that in general the operators with the low counts finished first, and those with the high last.

The detailed results of the second test are given in Table IV A, where it will be observed that each operator was very consistent. For example, operator T is higher on every plant but one than operator W, whose total is closest to his, likewise operator S is higher on every plant than operator M, and so on. Operator T with the highest count exceeded M, the lowest, by over 40 per cent. This is a large difference which could introduce serious errors into the method. However, the counts could be expressed relative to a standard or control set of plants. Plants Nos. 6 and 7, with the largest number of spots, may be considered the controls, and the results on the other plants have been expressed as a percentage of these, and appear in Table IV B. No significance can be shown now between the operators, though the differences between the plants remain. This method of presenting results will be discussed in more detail later. It is concluded that experienced operators differ significantly in their counts from one another and even from test to test, but within a test they are very consistent. Hence

if their counts are expressed on the basis of their own controls, appreciable operator errors will be eliminated.

VARIATION IN REPLICATE CONTROL PLANTS

It has been observed with these methods that the replicate control plants appear to vary more for some diseases than for others, when the factors discussed above were the same for the entire group of plants. One

TABLE IV

OPERATOR TESTS ON COUNTING EARLY BLIGHT LESIONS

A. Number of lesions counted per 2 leaves. Second test

Plant No.	Operators						Total
	T	W	A	C	S	M	
1	189	160	167	156	148	142	962
2	103	86	81	66	70	68	474
3	172	140	133	119	118	115	797
4	268	273	197	213	189	181	1321
5	242	203	216	200	184	177	1222
6	444	384	308	348	325	312	2121
7	304	249	260	226	239	230	1508
8	219	215	156	177	174	149	1090
Total	1941	1710	1510	1505	1447	1374	9495

B. Number of lesions as per cent of control plants

Plant No.	T	W	A	C	S	M	Total
1	51	51	59	54	53	52	320
2	27	27	28	23	25	25	155
3	46	44	47	42	42	42	263
4	72	86	69	73	67	67	434
5	65	64	76	70	65	65	405
8	59	68	55	62	62	55	361
Total	320	340	334	324	314	306	1938

measure of the precision attainable with a given disease is the uniformity in lesion counts for replicate control plants within the same test. Hence it is of interest to compare the variation in replicate controls for the different diseases. All of the available data for a period of six months were examined; the replicate checks comprised 2, 3, or 4 control plants per test. In the case of Late Blight there were 36 sets of replicate controls, totaling 107 plants, while for Early Blight there were 34 sets, totaling 96 plants, and for Septoria Leaf Spot 10 sets of duplicate controls. A preliminary examination indicated that the variance was related to the mean; hence the total number of lesions per plant of three leaves was converted to logarithms and the variance calculated for each set of replicate plants. These variances were tested for homogeneity within a disease by Bartlett's method (17, p. 206) and in all three cases were reasonably homogeneous.

However, when the three mean variances for each disease were compared, the χ^2 value for 2 D.F. was 38.3, showing clearly a lack of homogeneity. The standard deviations were derived from the mean logarithmic variances, transformed to antilogarithms and expressed as ratios, thus:

Replicate control plants	Mean logarithmic variance	Standard deviation
Late Blight	.00911	1.25
Early Blight	.01586	1.34
Septoria Leaf Spot	.08856	1.98

Since the variation in the number of lesions in replicate control plants of Late Blight is the least, it is to be expected that this would prove to be the most precise of the three diseases or methods, while Early Blight would be somewhat less precise, and Septoria Leaf Spot markedly less.

Septoria Leaf Spot was omitted from further consideration because of the greater variability of the control plants, as well as the slowness with which lesions develop and its property of spreading under normal greenhouse conditions.

FUNGICIDE TESTS

After the method had been reasonably well worked out and standard conditions arrived at, a special experiment was made on Early and Late Blights using nine different fungicides. The object was to furnish basic data for determining the most suitable manner of expressing results, the reproducibility of results from test to test, the most efficient number of plants per dose, the slope of the dosage response curve, the relative precision at different levels on the curve, and the comparative sensitivity of the two diseases to control by different types of fungicides.

PLAN OF EXPERIMENTS

The fungicides comprised three copper, three sulphur, and three organic compounds, namely Bordeaux mixture with equal copper sulphate and hydrated lime, Yellow Cuprocide,[9] and Copper Fungicide "34"[10]; Lime Sulphur,[11] Mike Sulphur,[12] and Dry Wettable Flotation Sulphur[13]; Wettable Grade Spergon,[14] Fermate,[15] and Thiosan.[16] The copper fungi-

[9] Cuprous oxide, 86% copper. Röhm & Haas Co., Inc., Philadelphia, Pa.

[10] Copper 34%. Tennessee Copper Co., Copperhill, Tenn.

[11] 32° Baumé. Andrew Wilson Inc., Springfield, N. J.

[12] Sulphur 95%. The Dow Chemical Co., Midland, Mich.

[13] Sulphur 95%. Koppers Co., Tar & Chemical Division, Pittsburgh, Pa.

[14] Tetrachloro-para-benzoquinone 50%. Control M 6922. United States Rubber Co., Naugatuck Chemical Division, New York, N. Y.

[15] Ferric dimethyldithiocarbamate 70%. E. I. du Pont de Nemours & Co. Inc., Grasselli Chemicals Department, Wilmington, Dela.

[16] Tetramethyl thiuramdisulphide 50%. Bayer-Semesan Co., Inc., Wilmington, Dela.

cides were applied at concentrations of 1.0, 0.2, 0.04, 0.008, and 0.0016 per cent total solids (Bordeaux, per cent copper sulphate), the sulphur fungicides at 5.0, 1.0, 0.2, and 0.04 per cent total solids (lime sulphur, per cent by volume of 32° Baumé stock), and the organic fungicides at 0.2, 0.04, 0.008, and 0.0016 per cent total solids. It will be noted that the dose ratio was 5 in all cases. A dosage of 1 per cent is approximately equivalent in the field to 8 lbs. per 100 gallons. All nine fungicides were tested at the same time against each disease; there were two plants at each dose and four control plants. The complete test was replicated three different times, a week to a month apart. The spore concentration for *Alternaria solani* was 40,000 per cc. and for *Phytophthora infestans* 10,000 per cc. Otherwise the standard conditions described above were maintained throughout.

METHODS OF EXPRESSING RESULTS

The results are first recorded as the number of disease lesions per plant of three leaves for each dose of the fungicide. In this form, however, they are not particularly illuminating. If the number of lesions is expressed as per cent of the check, that is, as per cent of infection, a value is obtained which not only expresses the per cent disease or inversely the per cent control, but also is relatively independent of fluctuations in the number of lesions in the checks from test to test as is illustrated in Figure 7.

These results were then plotted on logarithmic probability paper (20) and the dosage response curve obtained graphically. Individual curves were drawn for each of the duplicate plants and hence for the three replicate tests. Just prior to the time of spraying, the replicate plants were chosen at random, one set being labeled A, the other B; thus the two series were selected in advance of the results. Typical results for the six curves are given in Figure 11 where control of Early Blight by Thiosan and of Late Blight by Tennessee Copper "34" are illustrated. It will be noted that these greenhouse disease curves plot as straight lines on logarithmic probability paper. From such curves the LD50 and LD95 values were obtained graphically (12, 20) for all the copper and organic fungicides against both diseases. In greenhouse studies the LD95 may be defined as the dose preventing 95 per cent of infection. Hence the LD95 is the equivalent of 95 per cent disease control, or conversely 5 per cent disease.

Analysis of Variance of LD50 and LD95 Values

An analysis of variance was performed on these LD values expressed as the logarithm of parts per million, in order to ascertain the relative magnitude of the variation associated with replicate plants, replicate tests, LD levels, and diseases. The four analyses are given in Table V. In comparing fungicides, the logical error term to use is the fungicide × test interaction, since for two fungicides to differ significantly they must differ con-

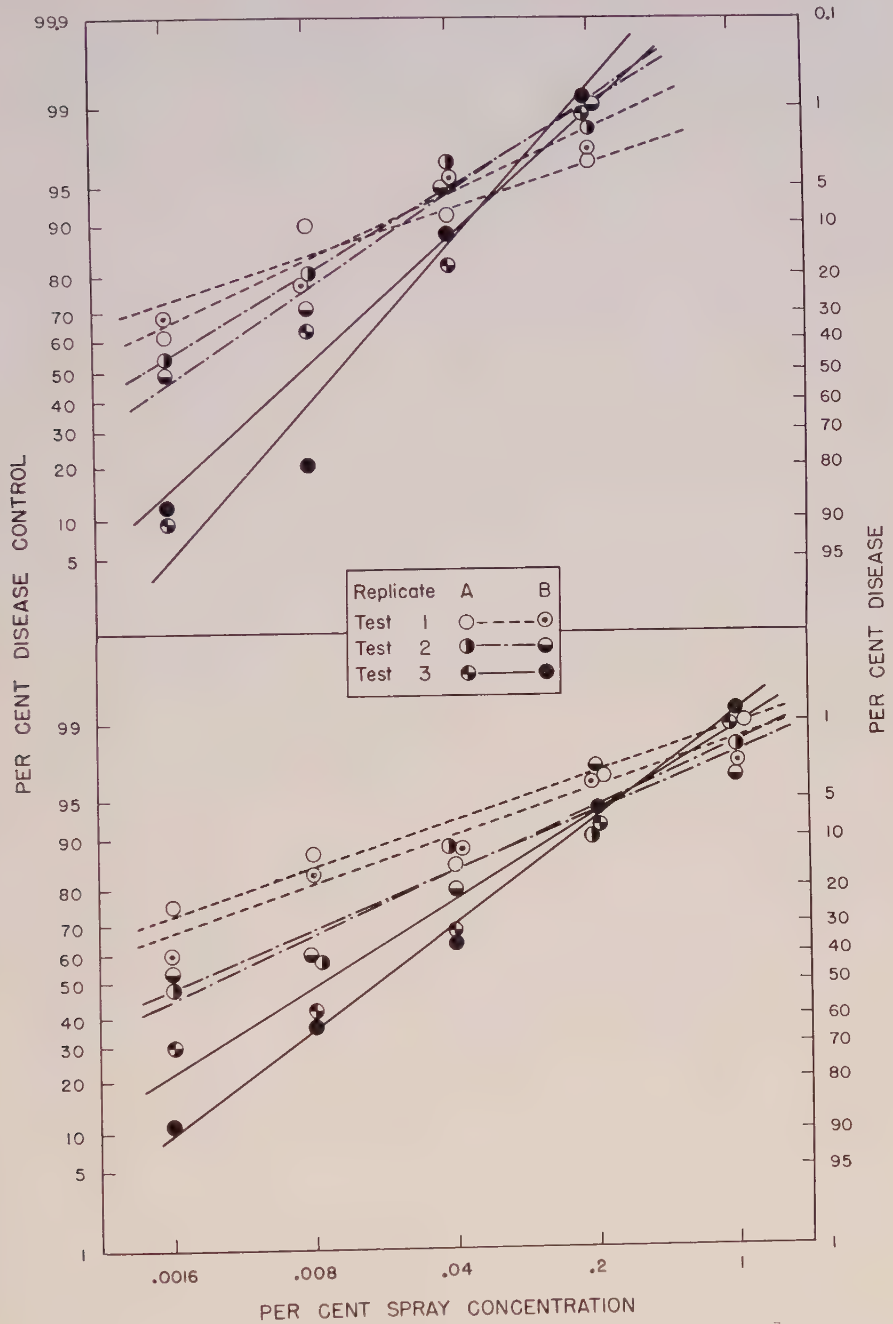

Figure 11. Typical greenhouse dosage-response curves. Top. Control of Early Blight by Thiosan. Bottom. Control of Late Blight by Tennessee Copper "34." Note variation in replicate tests or plants (A and B) diminishes as per cent disease control increases.

sistently from test to test (11). The replicate plant error will merely indicate which fungicide is better within a given test. Thus, in comparing the relative merits of the LD50 and LD95 for evaluating fungicides, preference would be given to the LD level showing the greatest ratio between fungicide variance and fungicide × test interaction. In both diseases it will be seen that the fungicide variance is greater and the fungicide × test interaction is lower at the LD95. The LD95 level also has the greater practical advantage of indicating a high degree of control. These analyses also show that the variation in replicate tests is highly significant, though it is

TABLE V

ANALYSIS OF VARIANCE OF LOGARITHMIC LD50 AND LD95 VALUES IN PARTS PER MILLION FOR COPPER AND ORGANIC FUNGICIDES AGAINST EARLY AND LATE BLIGHTS OF TOMATO

	D.F.	LD50		LD95	
		Variance	Significance	Variance	Significance
Early Blight					
Fungicides	5	1.884	High/FT	7.017	High/FT
Tests	2	2.181	High/FT	0.649	Sign./FT
Fungicides × Tests	10	0.208	High/RP	0.117	Sign./RP
Replicate Plants	18	0.048		0.046	
Late Blight					
Fungicides	5	0.272	No/FT	0.769	High/FT
Tests	2	3.927	High/FT	0.467	Sign./FT
Fungicides × Tests	10	0.193	High/RP	0.080	High/RP
Replicate Plants	18	0.026		0.011	

Error terms: FT = Fungicides × Tests; RP = Replicate Plants.
Significance: High = > 100:1; Sign. = 100:1 to 20:1; No = < 20:1.

reduced somewhat at the LD95 level. This may be observed graphically in Figure 11 by the decrease in spread between the replicate tests at the LD95 level as contrasted with the LD50. The replicate plant and the fungicide × test interactions are lower for Late Blight than for Early Blight, indicating that the former disease gives the greater precision. This is also substantiated by the significantly lower control replicate plant variance shown above for Late Blight. Since the replicate plants contribute so little to the total variance and since they cannot be used for error, it would be more efficient to run more tests with single plants in preference to fewer tests with replicate plants.

Comparison of Replicate Test and Replicate Plant Variances at Different Probit Levels

In view of the apparent greater precision at the LD95 level compared to the LD50 level, which is the reverse of laboratory tests, a further study

was made. All of the per cent disease data on the copper and organic fungicides, excluding that of Spergon which has a significantly steeper slope, were converted to probits and analyses of variance run within diseases, fungicides, and spray concentrations. In this manner a series of replicate test and replicate plant variances were obtained, each of the former having 2 degrees of freedom, and each of the latter 3 degrees of freedom. The variances were then grouped into classes according to the mean probit for the respective replicate plants or replicate tests. The probit classes were 2 to 3 (0.2 to 2.2 per cent disease), 3 to 4 (2.3 to 15.9), 4 to 5 (16.0 to 49.9), and 5 to 6 (50.0 to 84.0). Bartlett's χ^2 test for homogeneity of variances (17, p. 206) was then performed on all of the replicate test variances within each class, and also similar tests were performed on the replicate plant variances. The detailed results are shown in Table VI.

TABLE VI

Homogeneity of Variances of Replicate Plants and Replicate Tests at Different Probit Classes for Amount of Disease

Class		Mean variance	D.F.	χ^2	Probability
Probit	Per cent				
Replicate plants					
2–3	0.2– 2.2	.0532	21	9.18	.20–.10
3–4	2.3–15.9	.0492	36	8.31	.95–.50
4–5	16.0–49.9	.0896	45	22.80	.10–.05
5–6	50.0–84.0	.1752	33	5.20	.95–.50
All classes				16.80	<.01
Replicate tests					
2–3	0.2– 2.2	.0612	14	3.30	.95–.50
3–4	2.3–15.9	.1949	24	5.30	.95–.50
4–5	16.0–49.9	.4713	30	12.97	.95–.50
5–6	50.0–84.0	.9339	22	7.58	.95–.50
All classes				16.57	<.01

In all cases it was found that the variances within a probit class were homogeneous. However, when the variances from the different probit classes were combined, homogeneity could not be demonstrated for either replicate tests or replicate plants. That is, there is a significant difference in variance of replicate plants or replicate tests at different probit or percentage levels. These data indicate that for each successive decrease of one probit in amount of disease, to a minimum level, the variance is approximately halved. The minimum level for replicate plants appears to be attained at the probit value of about 3 with a mean variance of 0.05, while for replicate tests the apparent minimum is lower. In the orthodox use of

the probit transformation with percentage response data, the weight decreases on either side of probit 5 (LD50), so that a probit of 3.355 (5 per cent disease or LD95) has only 0.35 of the weight of a probit of 5. However, in the greenhouse methods where the probits represent the per cent of disease relative to the control, a probit of 3.355 would appear to have approximately one-third the variance of a probit of 5. Since the weight is equal to the reciprocal of the variance, the LD95 would be expected to have about three times the weight of the LD50. In Table V the LD50 replicate test and replicate plant variance for both diseases is greater than that of the comparable LD95 variance. The average ratio of LD50 to LD95 variance is 3.8.

Per Cent Disease at a Given Dose

In certain types of greenhouse comparisons, and presumably in some field comparisons, the use of dosages giving equal response, i.e., LD values, is unsuitable as a basis for evaluation of fungicides. If preliminary comparisons are made at single dosages of the chemicals, with the resultant saving in numbers of plants used, toxicity curves cannot be constructed and the dose for equal response cannot be estimated. In the case of relatively ineffective compounds or compounds which are phytotoxic, a high degree of control (LD95) either cannot be estimated or can be estimated only by extrapolation; further, under certain conditions, control with all concentrations tested may exceed the LD95.

An alternate method that obviates these difficulties is that of expressing results as the per cent disease or per cent infection at a given dosage. This may be done in preliminary work by testing all compounds at the same dosage or, in more precise comparisons, by using the desired dose as an intermediate value in a dosage series and obtaining the per cent disease from a toxicity curve on logarithmic probability paper. With steep toxicity curves, i.e., arithmic λ values (reciprocal of slope) (12) less than 2, this method is not applicable as most values obtained at any given concentration are either 0 or 100 per cent. However, with flat slopes, which are common with this technique as will be shown later, a large proportion of compounds may be expected to give a finite per cent disease when a suitable dosage is chosen. The relative precision of the LD95 or per cent disease depends on the slope of the toxicity curve; when the λ value is 10 (a slope of 1), they are equal in precision, as the slope becomes steeper the precision of the LD values increases, and as it flattens the precision of the per cent disease increases. In tomato foliage disease work with Late Blight and Early Blight 0.2 per cent concentration of spray is suitable for making per cent disease comparisons.

Comparisons of per cent disease values must be made using some function which gives all values equal precision in order that averages and

significant differences can be correctly computed. In order that the appropriate transformation be determined an experiment was conducted with Early Blight. By use of varying concentrations of Bordeaux mixture and Spergon widely differing per cent disease means were obtained. Six single plant replications of each treatment permitted the determination of the variance for each group of data in the arithmic form and in several common transformations; the results are presented in Table VII. It will be noted that means and variances were not determined by the equivalent angle and probit transformations in the first row of Table VII. They could not be obtained since individual determinations that composed this mean gave 100 per cent or more disease. This situation is due to experimental variation and cannot be handled in these two transformations since no equivalent angle or probit exists for 100 per cent or more. The homogeneity of the variances for the arithmic values and for each of the transformations was then determined by Bartlett's χ^2 test (17, p. 206). Table VII shows

TABLE VII

Homogeneity of Variances of Per Cent Disease Results with Early Blight in Several Transformations. Six Determinations for Each Mean

Arithmic		Equivalent angles		Probits		Square root		Logarithmic	
Mean	Variance	Mean	Variance	Mean	Variance	Mean	Variance	Mean	Variance
82.3	999	—	—	—	—	8.67	3.95	1.88	.0367
68.0	615	60.6	531	6.08	2.505	8.15	2.54	1.81	.0230
42.0	115	40.2	49.9	4.73	0.098	6.42	0.62	1.61	.0134
31.5	266	32.0	163	4.47	0.247	5.42	2.36	1.44	.0454
10.9	17.9	18.9	20.5	3.73	0.073	3.24	0.56	1.00	.0426
9.5	27.5	17.3	34.0	3.61	0.140	2.96	0.93	0.90	.0930
3.3	4.1	9.2	26.9	2.99	0.328	1.61	0.81	0.46	.0833
χ^2	41.83	χ^2	20.61	χ^2	20.43	χ^2	9.405	χ^2	8.073
Prob.	<.001	Prob.	.001	Prob.	.001	Prob.	.15	Prob.	.24

that neither the arithmic values nor the equivalent angle or probit transformations gave homogeneous variances while both the square root and logarithmic transformations give very reasonable probabilities for homogeneity of variances over the range covered in the experiment. It is suggested that the logarithmic transformation be used, as conversion tables are more readily available for it than for the square root transformation.

A logical explanation for the equalizing of variances by a logarithmic transformation may be shown by example: When control plants are varying from 75 to 150 lesions per plant with a mean value of 100, individual plants receiving completely ineffective treatments may be expected to vary in per cent disease recorded from 75 to 150 while a treatment that allowed a mean of only 2 lesions to develop could not be expected to vary from 0

to 75 lesions but instead between 1.5 to 3 lesions. In logarithms, these differences would be numerically equal ($\log_{10} 75 = 1.88$, $\log. 150 = 2.18$, and $\log. 1.5 = 0.18$, $\log. 3 = 0.48$, the difference between both pairs in logarithms is 0.30), consequently their variances should be equal. In Table VII it will be seen that the largest variances obtained are with the lowest logarithmic means noted; this may be strictly coincidental since the variances are homogeneous. It should be pointed out, however, that the size of the sample, i.e., the number of lesions counted, lessens as the mean grows smaller. Thus it is reasonable to expect this to play an important part with means of less than 10 and it is not to be assumed that a mean of 1 or less will have the precision attained with larger samples. When it is desired to compare extremes of per cent disease, for example, 100 per cent and 0.5 per cent, it must first be shown that their logarithmic variances are homogeneous.

It is to be noted that it is necessary to express results as per cent disease in order to achieve homogeneous variances in the logarithmic transformation; the inverse relation per cent disease control will not give homogeneous variances by this procedure. In certain figures which follow the dosage-response curves have been plotted on the basis of per cent disease control in order to follow the orthodox presentation of toxicity curves and to facilitate the determination of LD95 or LD50 values. If statistical comparisons are desired for response at a given dosage, it will be necessary to read the inverse, per cent disease as shown in Figure 11, and convert to logarithms. Although it has been shown that the variance of probits is not the same at different levels, calculations on LD95 or LD50 values may be made since the level for each is constant.

CONTROL OF DISEASES BY DIFFERENT FUNGICIDES

The results of the fungicide tests are summarized graphically in Figure 12. Each point is derived from the probit mean of the six replicate plants constituting the three replicate tests. The LD95 and the mean per cent disease at a concentration of 0.2 per cent spray are shown in Table VIII.

Relative Control

It will be noted that the organic fungicides gave good control and the copper fungicides fair control of both diseases, while the sulphur fungicides, excepting lime sulphur, were relatively ineffective. The poor showing of the sulphur compounds would be predicted from field experience. Actually lime sulphur injures tomato foliage in the greenhouse seriously at 5 per cent so that normal lesion counts cannot be made. Due to their ineffectiveness, or phytotoxicity, suitable data from the sulphur fungicides were thus not available for the analyses of Tables V and VI. The three organic fungicides gave better disease control than the copper fungicides;

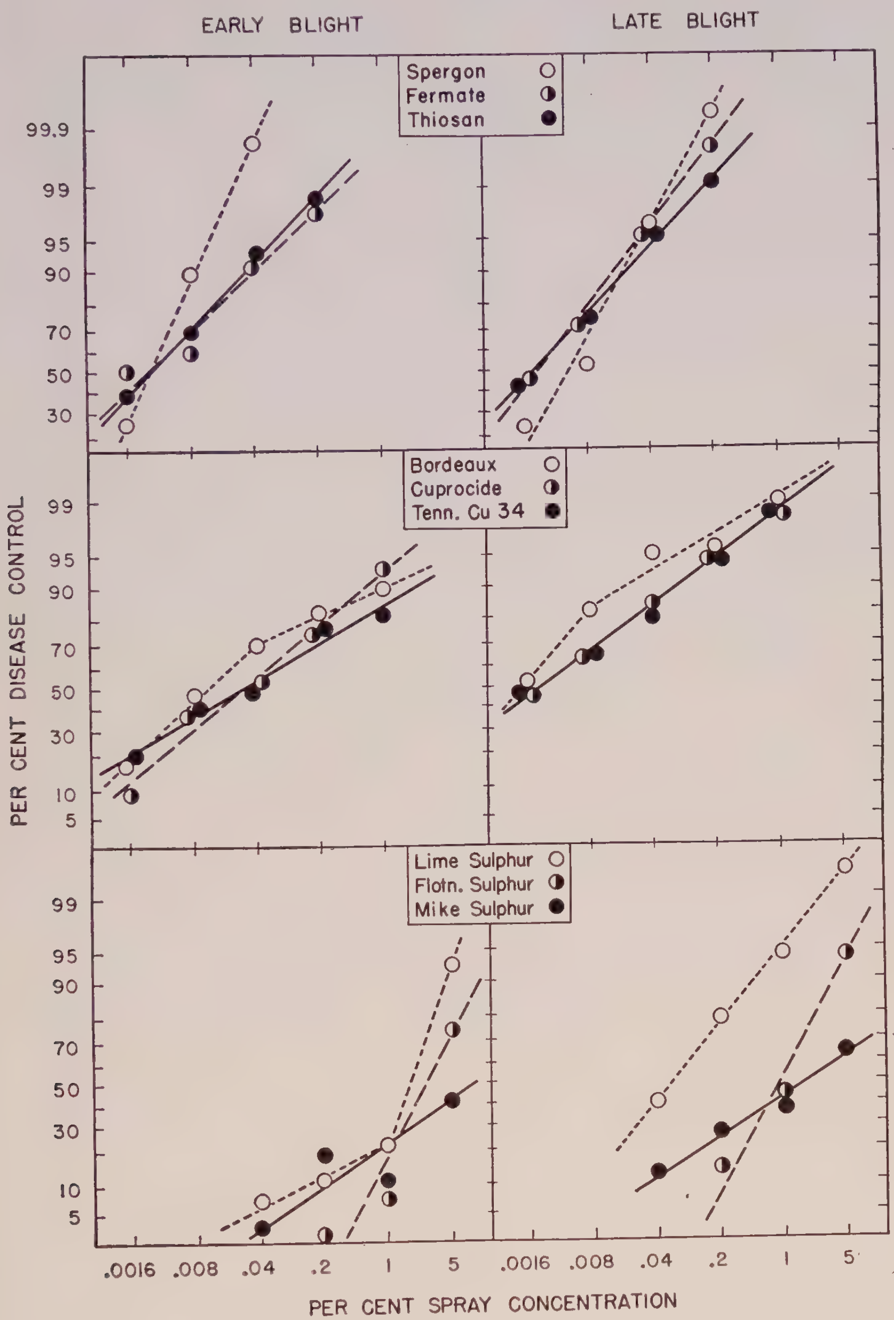

Figure 12. Dosage-response curves for organic, copper, and sulphur fungicides against Early and Late Blights. Note slopes and relative control. Each point mean of 3 tests with 2 plants each.

however, the sprayed plants were not subjected to a rain or other weathering test which might modify the results. The two diseases did not respond alike to the different fungicides; an analysis of variance on the combined LD95 values for Early and Late Blights, copper and organic fungicide data, showed a highly significant disease × fungicide interaction. As may be seen in Table VIII, the three organic fungicides gave good and equivalent control of both diseases, while the copper fungicides were very effective against Late Blight but only fair against Early Blight.

TABLE VIII

EFFECTIVENESS OF DIFFERENT FUNGICIDES AGAINST EARLY AND LATE BLIGHTS OF TOMATO

Fungicide	LD95 in %*	Per cent disease at 0.2% spray conc.*
Early Blight		
Bordeaux mixture	6.8	17.6
Yellow Cuprocide	1.8	21.8
Tennessee Cu "34"	9.4	28.3
Lime Sulphur	5.7	87.5
Mike Sulphur	>10.0	90.3
Flotation Sulphur	>10.0	>99.0
Spergon	0.012	< 0.1
Fermate	0.084	2.1
Thiosan	0.062	1.4
Late Blight		
Bordeaux mixture	0.086	2.8
Yellow Cuprocide	0.220	5.5
Tennessee Cu "34"	0.220	5.5
Lime Sulphur	1.0	23.3
Mike Sulphur	>10.0	76.4
Flotation Sulphur	5.4	92.5
Spergon	0.034	< 0.1
Fermate	0.033	0.2
Thiosan	0.046	0.8

* Per cent total solids, except Bordeaux—% copper sulphate and lime sulphur—% 32° Baumé stock.

Slope of Curves

As will be seen in Figures 11 and 12, the majority of the dosage response curves plot as straight lines on logarithmic probability paper. Only Bordeaux mixture against both Early and Late Blights appears to "break," giving a curve convex upwards (12). The variance of the points showing less than 16 per cent disease control (84 per cent disease or probit 6) be-

comes so great that the slope in this range cannot be determined with any significance. The slopes with all compounds examined are flatter in the greenhouse than in the laboratory as shown by the examples in Figure 13. However, there are large differences in the steepness of slope in the greenhouse: Spergon, Lime Sulphur, and Flotation Sulphur have approximate arithmic λ values of 3; Fermate and Thiosan have λ values of 7; and Bordeaux, Yellow Cuprocide, Tennessee Copper "34," and Mike Sulphur have λ values averaging about 25. These slopes have not been compared by orthodox statistical methods (2, 3) since it has been demonstrated that the usual method of weighting for probits does not hold for these disease control curves. It may be noted that the three classes of slope steepness are not correlated with the three logical fungicide groups, namely copper, sulphur, and organic. However, the two diseases did give the same slope with the same fungicide, indicating for these data that the slope was more dependent on the fungicide than on the disease. A similar conclusion was reached for laboratory tests on fungus spores (12).

In a previous laboratory study (9) it was noted that fungicidal curves are in general flatter than fungistatic. Hence it may be significant that these greenhouse curves are flatter than in the laboratory and indicate that their action is fungicidal rather than fungistatic.

COMPARISON OF RESPONSE OF A SPECIFIC FUNGUS TO SIMILAR FUNGICIDES IN LABORATORY AND GREENHOUSE TESTS

Absolute comparisons of laboratory dosage-response curves with those of the greenhouse or field have never been made. Accordingly an experiment was designed in which the same fungicides would be tested against a specific fungus at a constant spore concentration for both laboratory slide-germination and greenhouse methods. The Early Blight organism, *Alternaria solani* (Maine strain 52), is one of the few suitable for such a comparison. Accordingly spores of this fungus were obtained under standard conditions given above and subjected to a settling tower test (14) with the copper fungicide, Tennessee Copper "34," and the organic, Thiosan. It is necessary that the spore concentration be comparable for both methods. The greenhouse tests on Early Blight were performed at a spore concentration of 40,000 per cc. Under standard conditions, approximately 17 spores are deposited per sq. cm. of leaf surface per stock concentration of 10,000 per cc. (see page 104), hence 40,000 would deposit about 70 per sq. cm. It has been shown for laboratory tests that 50,000 spores per cc. are equivalent to 35 spores per low power field of 1.3685 sq. mm. (12) or approximately 25 spores per sq. mm., or 2,500 per sq. cm. Hence, in order to obtain a spore deposit of 70 per sq. cm. in the laboratory it would be necessary to have a concentration of $(50{,}000 \times 70)/2{,}500$ or 1,400 spores per cc. This concentration is too low for testing in the labora-

tory with any precision or efficiency. However, laboratory tests were made at concentrations of 50,000, 16,700, and 5,600 against both Tennessee Copper "34" and Thiosan and replicated. The results showed that a linear relationship held between the logarithm of the spore concentration and the logarithm of the fungicide deposit at any LD level. This correlation indicated that the same response would result from 1,400 spores per cc. at 0.35 dosage as at 50,000 spores for a dosage of 1.

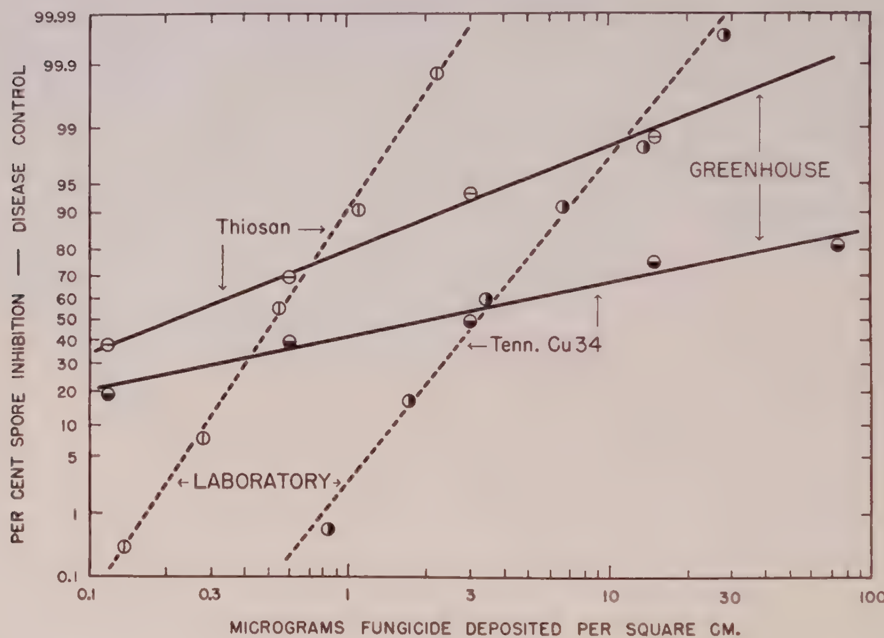

FIGURE 13. Absolute comparison of laboratory and greenhouse dosage-response curves; identical fungus and fungicides. Tennessee Copper "34" and Thiosan against Early Blight (*Alternaria solani* Maine strain). Spore concentration constant, 70 per sq. cm.

The dosage response curves resulting from the mean of three different tests at 50,000 spores per cc. were thus adjusted on the dosage axis to the equivalent of 1,400 spores per cc. and are plotted in Figure 13. In the same figure there is also shown the mean of three tests on Early Blight as obtained from the data of Figure 12, but plotted on a basis of micrograms fungicide deposited per sq. cm. Thus on one graph there is shown the mean of three laboratory and three greenhouse tests of Tennessee Copper "34" and Thiosan against respectively the Early Blight organism and disease at the same spore load of 70 per square centimeter, and comparable fungicide deposit.

It will be observed in Figure 13 that over the middle range, i.e., ap-

proximately LD_{50}, the dosage is the same for the laboratory and greenhouse methods. This is perhaps unexpected and coincidental, in that the former test was performed on glass slides and the latter on the leaf surface of the natural host. As was shown above, the greenhouse curves are much flatter than the laboratory, though both give straight lines when plotted on logarithmic probability paper. In this particular case Thiosan gives a steeper curve in both laboratory and greenhouse than Tennessee Copper "34," though the difference between laboratory and greenhouse slopes appears to be similar for both fungicides. Also in this instance, Thiosan would be rated the better fungicide in both laboratory and greenhouse tests. In another paper (19) many fungicides are correlated as to rating in laboratory and greenhouse tests.

EXPERIMENTAL DESIGN

On the basis of these studies, general conclusions may be drawn regarding an efficient experimental design.

SUITABILITY OF DISEASES

Since different diseases rate fungicides differently, it is desirable to employ at least two diseases, preferably caused by organisms taxonomically widely separated. Early and Late Blights of tomato have proven satisfactory in all respects and are recommended. Septoria Blight is somewhat unsatisfactory in that the disease lesions require ten days to develop and the disease spreads in the greenhouse. If desired, it may be used to supplement the two main diseases.

STANDARD CONDITIONS

Standard conditions are essential for a reproducible and precise method of testing fungicides. The following standards adopted for the equipment described above are briefly enumerated.

Plants. Tomato var. Bonny Best, six inches tall. One plant per fungicide dose.

Spraying. Paint spray gun at 40 lbs. pressure, 30 seconds exposure, plants on turntable 50 inches from nozzle of gun.

Dosages. Preliminary tests—0.2 per cent stock concentration. Toxicity curves, three or more of the following concentrations: 0.0016, 0.008, 0.04, 0.2, 1.0, and 5.0 per cent fungicide.

Fungus spores. Alternaria solani grown on potato dextrose agar Petri plates, spores 3 to 10 days after scraping, at a concentration of 25,000 per cc. *Phytophthora infestans* cultured on potato slices. Swarmspores obtained from sporangia held 6 hours at 10° C.; concentration of 5,000 per cc.

Inoculation and infection. Inoculation by hand atomizer at 20 lbs. pressure. Plants held in high humidity infection chambers 24 hours.

Counting lesions. Time—approximately 2 to 3 days after inoculation. Number of leaves—all lesions on leaves Nos. 4, 5, and 6, or 5, 6, and 7, which ever averages highest in check plants.

Check plants. Four per test; each operator to count all checks.

EVALUATION OF RESULTS

The total lesions per plant of three leaves are converted to a per cent of check; by so doing much of the day-to-day and individual operator variation in number of lesions is corrected. The per cent disease or conversely the per cent disease control is plotted on logarithmic probability paper and the LD95 or dose giving 95 per cent control is determined. Due to the flat dosage response curve the results may also be given as per cent disease at a given dosage.

Replicate tests. In the cases of a preliminary rating, a single dose repeated in a second test is sufficient, and a dosage of 0.2 per cent fungicide is suggested for this purpose. For precise comparisons, this upper portion of the dosage response curve should be determined which would require three or four doses. This test should be replicated at least twice or more, depending on the importance attached to the comparison.

Significant differences. LD95 values may be compared by means of the t test or F test with single degrees of freedom, the fungicide $\times$ replicate test interaction being used as error. If the method has become stabilized, a cumulative error term derived from accumulated tests may be used, as described in another article (11). Such an error term will tend toward infinite degrees of freedom and hence smaller differences can be shown with significance. However, the variance of the error of each new test must be shown by Bartlett's test (17, p. 206) to be homogeneous with the general error term. Due to the introduction of another variable, the plant and possibly other factors associated with the infection phenomena, the greenhouse method is not as precise as the laboratory method. The flatter dosage-response curves of the greenhouse, resulting perhaps from these factors, would not be expected to give as precise LD95 values as the steep laboratory curves (11, 12). From the data of Table V and the cumulative error shown elsewhere (11), it is to be expected that about a five-fold difference in LD95 values could be shown significantly in two tests, a three-fold difference in four tests, and approximately a 70 per cent difference in ten tests.

SUMMARY

1. The necessity of and requirements for a satisfactory greenhouse method of testing foliage fungicides are set forth. The tomato is considered an ideal plant and the important and representative diseases, Early Blight, Late Blight, and Septoria Leaf Spot, were selected for study.

2. The plants are sprayed within a hood on a compound turntable designed for one, two, three, or four plants. The fungicide is applied with an adjustable paint spray gun supplied with constant air pressure.

3. Tomato plants were measured as to leaf area relative to leaf position and plant size. The quantity of spray deposited on the leaves was determined for different variables, and the following were chosen as standard: 40 lbs. pressure, 2-1/2 fluid valve turns, 30 seconds spraying time, and 6-inch plants. Under these circumstances for a 3 plant turntable 0.75 cc. of spray is deposited per 100 sq. cm. leaf surface.

4. After spraying and drying, the plants may be placed on the turntable and subjected to an artificial rain of tap water delivered through a fine rose type nozzle.

5. The tomato plants are placed on the turntable and inoculated with a known concentration of spore suspension obtained by recognized cultural methods. The suspension is applied by means of a hand atomizer at 20 lbs. pressure for 30 seconds. Under these conditions an average of 17 spores are deposited per sq. cm. of leaf surface per concentration of 10,000 spores per cc. Immediately after atomizing the plants are placed in an incubation chamber, in which 100 per cent relative humidity is maintained by means of air conditioning jet nozzles. After 24 hours in the chambers the plants are returned to the greenhouse.

6. Lesions of Early and Late Blights develop within two or three days, Septoria Leaf Spot requires ten days. Late Blight lesions show fimbriated margins and must be counted before coalescing. The other two diseases show more definite lesions and do not coalesce readily. The total lesions on three leaves are counted.

7. Fifteen varieties of tomato were examined regarding growth habit, susceptibility to the diseases, type of lesions, and commercial importance. Bonny Best was selected as suitable for this method.

8. The number of lesions developing on different size plants and leaf positions was determined. On 6-inch plants the greatest number of lesions usually appears on leaves Nos. 5, 6, and 7. Per unit area basis, young plants and young leaves are more susceptible than older ones; the relation between number of lesions and leaf position is linear.

9. The maximum number of *Phytophthora infestans* motile swarmspores occurs after about six hours at 10° C. The number of lesions developing is directly proportional to the number of motile swarmspores. *Alternaria solani* spores show their maximum infectivity at from 3 to 10 days after scraping the mycelium. Spores of *Septoria lycopersici* are produced in abundance and infect readily from cultures several weeks old. These lesions produced by these two species are also directly dependent on the spore concentration. Approximately 200 lesions per three leaves may be expected to result, under standard conditions, from inoculating

with 5,000 spores per cc. of *Phytophthora*, 25,000 of *Alternaria*, and 200,000 of *Septoria*. The approximate per cent infection by the spores of three fungi is respectively 6.5, 1.7, and 0.2.

10. Experienced operators differ in their counts of number of lesions, but each is consistent within a given test, so that if the results are expressed as per cent of the check the operator error is reduced to non-significance.

11. The standard deviation for lesions on replicate check plants expressed as a ratio is: Late Blight 1.25, Early Blight 1.34, and Septoria Leaf Spot 1.98; these differ significantly.

12. A study was made of three copper fungicides (Bordeaux, Yellow Cuprocide, and Tennessee Copper "34"), three sulphur fungicides (Lime Sulphur, Mike Sulphur, and Flotation Sulphur), and three organic fungicides (Wettable Spergon, Fermate, and Thiosan). These were tested at three different times against both Early and Late Blights. Dosages of 0.0016, 0.008, 0.04, 0.2, 1.0, and 5.0 per cent concentration were applied to each of two plants. The lesion counts were converted to per cent of check and the results plotted on logarithmic probability paper.

13. An analysis of variance on the LD50 and LD95 (95 per cent disease control) values showed that the differences between replicate tests were highly significant but replicate plants contributed little to the total variation. The variance at the LD95 level is significantly less for both of the factors but greater for fungicides in the case of both diseases. A test for homogeneity of variances of replicate tests and plants at different LD or probit levels substantiated this and demonstrated that the LD95 is a more precise point to compare fungicides under these conditions than is the LD50. The variance is reduced progressively for each probit decrease in amount of disease, down to a probit of about 3.

14. The use of per cent disease in comparing the effectiveness of compounds is advocated for those situations where the use of LD95 values is not desirable. A suitable dosage, 0.2 per cent concentration of spray with tomato foliage diseases, will give finite per cent disease with a large majority of compounds and, in preliminary comparisons, furnishes maximum information with a limited number of plants. The use of the logarithm of per cent disease gives homogeneous variances and is thus adapted to statistical treatment.

15. All fungicides excepting Bordeaux, which was convex upward, plotted as straight lines on logarithmic probability paper. In general, these are much flatter than for the laboratory. Due to this flatness, the per cent disease at a given dose may be obtained with some precision. For both diseases, curves of Spergon, Lime Sulphur, and Flotation Sulphur were relatively steep, Fermate and Thiosan intermediate, and the copper fungicides and Mike Sulphur very flat.

16. A direct comparison was made in the laboratory of the same fungicides, Tennessee Copper "34," and Thiosan, against the same organism, *Alternaria solani*, at the same spore concentration. In this instance, at the LD50 level the dosages were the same for laboratory and greenhouse methods. Thiosan gave steeper dosage-response curves and rated the better fungicide by both methods.

17. It is concluded that Late and Early Blights are satisfactory in all respects as greenhouse test diseases, and both should be used; Septoria Leaf Spot is less satisfactory. The standard conditions outlined above are recommended. Preliminary tests may be made at one dosage, 0.2 per cent concentration of spray, on one plant, and repeated a second time. Precise comparisons should include a series of dosages at one plant each, repeated several times with a determination of the LD95. The error term is the fungicide $\times$ replicate test interaction. If specified conditions are met a cumulative error term may be used. A five-fold difference may be shown in two tests, a 70 per cent difference in ten tests.

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CUMULATIVE ERROR TERMS FOR COMPARING FUNGICIDES BY ESTABLISHED LABORATORY AND GREENHOUSE METHODS

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In evaluating fungicides in laboratory or greenhouse tests the problem arises as to the choice of a suitable error term. Two questions may be asked. What source of variation constitutes a valid error term, and in the case of a known method, can previous experience be used to advantage?

SOURCES OF VARIATION IN FUNGICIDE TESTS

In the bioassay of drugs where the potency of an unknown is to be compared to a standard, it is customary to make a precise comparison of the two dosage-response curves as determined by a single test (1, 2). However, in fungicide tests there is an interest in comparing a large number of chemically heterogeneous compounds; also the test organisms are available in abundance. Fungicides have thus been compared in replicated tests, i.e., tests at different times, and it has been shown that they may be rated differently in different tests (5). This is due largely to the differences in slope of the heterogeneous group of compounds. Hence the use of the internal error of a given dosage-response curve, as would normally be used in a bioassay of drugs, is of little practical importance in comparing fungicides. Instead the LD50 or LD95 values are obtained for different fungicides in different tests. These values may be determined satisfactorily by graphic means (5, 10). The LD50 is the most precise point for laboratory comparisons and is usually recommended, especially for a series of closely related or homogeneous compounds. However, in the case of greenhouse methods, where the amount of disease is expressed as per cent of the check, the LD95 has been shown to be the more precise point for comparison (4). In addition, the LD95 is of greater practical interest.

The LD50 or 95 values thus obtained may be compared by means of an analysis of variance, or by the t test (5, 9). If the LD values differ widely, ten-fold or more, it is desirable to use a logarithmic transformation (5, 9) before performing an analysis of variance. In such fungicide tests the primary variates will be (a) fungicides, (b) replicate tests, (c) replicate spore counts or replicate disease counts or replicate plants, i.e., replicated counts on spores or plants within a given test at a given time, and (d) fungi or diseases, provided two or more different fungi or diseases are being studied. In addition, variance will be contributed from the various possible double and triple interactions.

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Which of these constitutes a logical error term will depend on the information desired. If the interest is merely in comparing the fungicides as they performed against a single fungus or disease and in a single test, the replicate spore count or the replicate plant respectively may be used. In well controlled techniques these factors will contribute but little to the total variation, in fact, the variation of replicate spore counts may be no greater than that of random sampling (6). The triple interaction, fungicides×fungi (or diseases)×replicate tests, frequently cannot be shown to differ significantly from the replicate count variable and thus may be employed as error term under similar circumstances. However, the use of such an error term is not ordinarily recommended for fungicide comparisons because the information provided is of little value.

More frequently, it is desired to predict the performance of the fungicides in other tests or with fungi or diseases in general. Thus the fungicide ×replicate test interaction will allow for the determination of significant differences in fungicides over the variation to be expected in replicate tests, and will be based on the mean values for all fungi or diseases examined. Similarly the fungicide×fungus (or disease) interaction will show differences in the mean replicate test values over variation in behavior resulting from the use of different fungi or diseases. If the greatest of these double interactions is selected as the error term, the fungicides will be rated on the basis of performance under all conditions tested. Hence one fungicide will have to be consistently better than another under these conditions to show a significant difference. This will ordinarily be the fungicide×fungus (or disease) interaction.

However, it may be desired to ascertain how the fungicides react against a given fungus or disease in which case the fungicide×replicate test interaction within fungi or diseases would be employed, or in case only one fungus or disease were used, the simple fungicide×replicate test interaction. In field tests due to great changes in weather, the comparable interaction fungicides×tests or fungicides×years may not be suitable. Likewise, in field tests in different localities the fungicide×locality interaction may be very great, so that the use of either one of these interactions would fail to show any significant difference in the fungicides. In such cases it may be desirable to use the orthodox replicate count or triple interaction, though the use of a year or locality interaction with fungicides would provide a much wider basis for comparison.

A significant difference between fungicides having been shown in the analysis of variance, it may be desired to make individual comparisons either by means of the *t* test or by the single degrees of freedom method (9). However, caution should be exercised here as emphasized by Fisher (3) and Snedecor (9). In group comparisons by the single degrees of freedom, the individual items tend to reinforce one another, hence only natural groups should be made.

CUMULATIVE ERROR OF ESTABLISHED METHODS

When comparing fungicides by means of the t test, the least significant difference of means may be determined by the usual formula: LSD $= t_{(.05)} \times \sqrt{2V/k}$, where V is the error variance and k the number of determinations of LD values for each fungicide. The value for t at the .05 point is read from the t table with n equal to the degrees of freedom in the error term. Likewise, in a single degrees of freedom comparison, the n_2 value to use for entering the F table would be that corresponding to the degrees of freedom for error. When the degrees of freedom for the error term are few the t or F values are high and only very large differences between fungicides can be shown to be significant. A penalty has been exerted for having only a small amount of data and thus an inadequate estimate of the variability of the population. If more information were available, presumably smaller significant differences between fungicides could be shown. For example, with 2 degrees of freedom for error the t value at 5 per cent is 4.303, whereas at 100 degrees of freedom it is 1.984. Thus it will be seen from the above formula that a least significant difference of less than half would be required in the latter case.

In the case of highly developed standardized methods experience has shown that the variance of the error term is, under specified conditions, fairly constant (5). That is, from considerable experience (8) the standard deviation of the population has become known within narrow limits.

In order to profit by this accumulated experience it is necessary to demonstrate that the error variance of a particular small test or sample could have been drawn from the same population as the established error of the method. This may be done by means of Bartlett's χ^2 test for homogeneity of variances (9, p. 206). If homogeneity is thus demonstrated, the LD values in the specific case may be compared by the t test or by single degrees of freedom using the cumulative error term with its associated degrees of freedom. As data are accumulated, the degrees of freedom or n for the error term will of course approach infinity, though little is to be gained beyond 100 degrees of freedom. Thus comparisons may be made under the most favorable circumstances for showing a significant difference between fungicides.

Table I has been prepared to facilitate the χ^2 test for homogeneity of variances. It has been calculated, in part graphically, by Bartlett's formula and gives the necessary ratio between the error variances of the particular test or sample and that of the standard or established method in order to establish homogeneity within the 5 per cent limit. For example, the error variance of the standard method is 6 with 100 degrees of freedom, a particular test with 2 degrees of freedom has an error of 18; are these variances homogeneous? Entering Table I with 100 degrees of freedom for the smaller variance and 2 for the larger variance, we find that the limiting ratio is 5.0. In the example the ratio of larger to smaller variance is 18/6

TABLE I

ERROR VARIANCE RATIO OF SAMPLE AND STANDARD AT 5 PER CENT LIMITS FOR HOMOGENEITY

Degrees freedom		Larger variance												
		1	2	3	4	5	6	8	10	14	20	40	100	1000
Smaller variance	1	1270	697	588	544	521	507	493	483	474	467	461	456	450
	2	71.6	42.2	35.0	31.9	30.3	29.1	27.9	27.1	26.3	25.8	25.1	24.8	24.6
	3	31.7	19.1	15.7	14.2	13.3	12.8	12.1	11.7	11.3	11.0	10.6	10.4	10.2
	4	22.2	13.2	10.8	9.7	9.0	8.6	8.1	7.8	7.5	7.2	6.9	6.7	6.6
	5	17.9	10.8	8.7	7.7	7.2	6.8	6.3	6.1	5.8	5.6	5.3	5.2	5.1
	6	15.6	9.3	7.5	6.7	6.2	5.8	5.4	5.2	4.9	4.7	4.5	4.3	4.2
	8	13.2	7.9	6.3	5.6	5.1	4.8	4.4	4.2	4.0	3.8	3.6	3.4	3.3
	10	12.0	7.1	5.7	5.0	4.6	4.3	3.9	3.7	3.5	3.3	3.1	2.9	2.8
	14	10.7	6.3	5.1	4.4	4.0	3.7	3.4	3.2	3.0	2.8	2.6	2.5	2.4
	20	9.9	5.8	4.6	4.0	3.6	3.4	3.1	2.9	2.6	2.5	2.3	2.1	2.0
	40	9.0	5.3	4.1	3.6	3.2	3.0	2.7	2.5	2.3	2.1	1.9	1.7	1.6
	100	8.5	5.0	3.9	3.4	3.0	2.8	2.5	2.3	2.1	1.9	1.7	1.5	1.4
	1000	8.3	4.8	3.8	3.2	2.9	2.6	2.4	2.2	2.0	1.8	1.5	1.3	1.1

or 3, hence the variances could have been drawn for the same population. Thus the means of LD values of the small test may be compared using the error of the standard method and 100 degrees of freedom. It is also possible for the error of the sample to be less than that of the standard, for instance, in the above example suppose the sample error variance were 1; the ratio would then be 6. In this case, the table is entered at 2 D.F. for the smaller variance and 100 D.F. for the larger, whence a limiting value of 24.8 is found. Since 24.8 is larger than 6, it is concluded the variances are homogeneous. When homogeneity has been demonstrated, the cumulative error should be used even though in some cases it may be greater than that of the sample.

TABLE OF ESTABLISHED ERRORS

Data accumulated over a period of several years in this laboratory on certain specified techniques have been studied regarding the homogeneity of the fungicide×replicate test interaction within fungi or diseases. The data were limited to water soluble compounds or "insoluble" fungicides of such a physical nature that no gross mechanical errors are introduced in the preparation of replicate samples; this would include most commercial fungicides. Due to the large differences in effectiveness of fungicides and hence in their LD values, all the analyses were calculated on logarithms of the original values. This transformation also serves the purpose of making the error terms more widely applicable.

The laboratory method covers the test tube dilution technique (5, 6) and the settling tower technique (7) on LD50 values without a rain test. No consistent difference could be shown between the fungicide×replicate test variances with the fungi, *Sclerotinia fructicola* (Wint.) Rehm., *Alternaria solani* (Ell. & Mart.) Jones & Grout, Delaware strain, *Macrosporium sarcinaeforme* Cav., *Glomerella cingulata* (St.) Sp. & von S., *Botrytis* sp. (*cinerea* type), and *Rhizopus nigricans* Ehr., +strain, so the results were pooled. Three groups of compounds have been made as follows: A, homogeneous compounds, i.e., alike or closely related chemically, with steep dosage-response slopes; B, heterogeneous or dissimilar compounds with steep slopes less than 2λ (arithmic); and C, heterogeneous compounds with flat slopes greater than 2λ, or compounds, some having flat slopes and others having steep slopes. Data were not available on homogeneous compounds with flat slopes. The reciprocal of the slope, λ, is determined graphically on logarithmic probability paper from the ratio LD84/LD50 expressed arithmetically (5, 10). All the fungicide×replicate test variances for A class were found to be homogeneous by Bartlett's method. However, those in B and C classes were not entirely homogeneous as might be expected from heterogeneous compounds, but the difference between the classes was highly significant. A summary of these data on cumulative

error terms is given in Table II together with the necessary differences required for significance, for a given number of tests. Since these values were determined on logarithms and converted to antilogarithms, the differences required for significance are in reality ratios.

TABLE II

FUNGICIDE × REPLICATE TEST CUMULATIVE ERROR VARIANCES FOR SPECIFIED LABORATORY AND GREENHOUSE METHODS, TOGETHER WITH DIFFERENCES REQUIRED FOR SIGNIFICANCE AT 5 PER CENT POINT BETWEEN FUNGICIDE MEANS

Method*		Fungicide × replicate test error	
		D.F.	Variance (log.)
A	Laboratory, Similar compounds Steep slopes	54	0.0042
B	Laboratory, Dissimilar compounds Steep slopes	322	0.0170
C	Laboratory, Dissimilar compounds Flat slopes	480	0.0480
D	Greenhouse, Early and Late Blights All compounds	321	0.1170

Ratio for Significant Differences Between Means

Method	Number of tests				
	2	3	4	6	10
A	1.35	1.28	1.24	1.19	1.14
B	1.81	1.67	1.52	1.41	1.20
C	2.70	2.25	2.02	1.77	1.37
D	4.71	3.54	2.99	2.45	1.67

* See text for details.

Greenhouse data were obtained for Early and Late Blights of tomato, for homogeneous and heterogeneous compounds, and for LD95 values derived from several different dosages and for per cent disease at one concentration, i.e., 0.2 per cent (4). The variances of all were found to be homogeneous and are likewise summarized in Table II. The relatively flat slopes of the greenhouse method together with its lower precision do not allow fine distinctions to be made among this collection of miscellaneous data. It is to be noted, however, from another study (4) on a single group of compounds, that the Late Blight method is somewhat more precise than the Early Blight.

It will be seen in Table II that homogeneous compounds in the laboratory, having similar steep slopes and composition, can be compared with greatest precision, while greenhouse methods though of perhaps more practical interest are much less precise. This perhaps is due to the introduction of another variable, the host plant, and in association with it the

phenomenon of infection. It is probable that under field conditions of comparable design, even greater differences would be required for significance.

SUMMARY

The fungicide$\times$replicate test interaction is suggested as the error term for laboratory and greenhouse methods of testing fungicides. With well developed and standardized methods this error term is reasonably constant and known. One handicap of small tests, that of inadequate knowledge of the standard deviation, may be overcome if it can be demonstrated by means of Bartlett's χ^2 test that their error variance is homogeneous with that of the known method. In this case t or single degrees of freedom comparisons are made with the cumulative error term and its larger degrees of freedom. A table is given showing the limiting ratios of error variance of sample to that of standard necessary to demonstrate homogeneity within the 5 per cent limit. Established error terms for certain specified laboratory and greenhouse methods are given together with the necessary differences for a given number of tests.

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CORRELATIONS WITHIN AND BETWEEN LABORATORY SLIDE-GERMINATION, GREENHOUSE TOMATO FOLIAGE DISEASE, AND WHEAT SMUT METHODS OF TESTING FUNGICIDES[1]

R. H. Wellman[2] and S. E. A. McCallan

Considerable time and effort have been spent on the development of the slide-germination method of testing fungicides (2) and a previous paper (7) has been devoted to the work done on greenhouse methods. However, little attention has been paid to the correlations between different methods of testing fungicides, even the work on comparison of results with different laboratory methods is limited; and no papers have appeared on the correlation between the initial laboratory slide-germination method and various greenhouse methods. Since the object of all fungicide testing is to predict the performance of compounds under specified field conditions, it is essential that comparable methods of testing be highly correlated with field results and, therefore, with each other. Only preliminary information is available on the behavior of compounds of different chemical types in the field but it is hoped that, at some future date, ample field data will be obtained for correlation with laboratory and greenhouse results. Considerable information has been obtained that is suitable for determining the correlations within and between the laboratory slide-germination and the tomato foliage disease techniques. Less precise data are available for control of stinking smut of wheat by a series of compounds. It is the purpose of this paper to present the correlations obtained with these three methods.

METHODS

TESTING TECHNIQUES

Slide-germination. The slide-germination (laboratory) method (2) was used for the determination of the fungistatic (6) LD50 values for a total of 599 compounds with four fungi. Essentially this method consists of placing a known concentration of spores in continued contact with a given dosage of chemical and observing germination after 24 hours. Some compounds were applied by the settling tower technique (2) and others examined by the test-tube dilution technique (2). The fungi used with this method were: *Sclerotinia fructicola* (Wint.) Rehm., *Glomerella cingulata* (St.) Sp. & von S., *Alternaria solani* (Ell. & Mart.) Jones & Grout, and *Macrosporium sarcinaeforme* Cav. These fungi are designated throughout the paper by their generic names.

[1] A preliminary report on this paper was submitted for presentation before the American Phytopathological Society, New York, N. Y., December, 1942 (12).

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Tomato foliage diseases. The tomato foliage disease (greenhouse) method (7) was used to determine the per cent disease response, following sprays with 0.2 per cent concentrations (7) of 56 compounds, for three diseases. In brief, single tomato plants are given a known dosage of chemical applied as a spray, are allowed to dry, atomized with a given concentration of spores, placed immediately in high humidity chambers and held there 24 hours to allow for infection, and removed to the greenhouse to develop lesions when counts are made on three comparable leaves on each plant. The variety Bonny Best of tomato (*Lycopersicon esculentum* Mill.) was used in all foliage disease work and the three foliage diseases were: Early Blight caused by *Alternaria solani*, Late Blight caused by *Phytophthora infestans* (Mont.) de Bary, and Septoria Leaf Spot caused by *Septoria lycopersici* Speg. Different strains of *A. solani* were used in the laboratory and greenhouse since one is not pathogenic and the other does not produce the abundance of spores necessary in the laboratory procedure. The strain used in the laboratory is specifically described in footnote 5 of another publication (9, p. 50), and that used in the greenhouse in (7, p. 102).

Wheat smut. An adaptation of tentative standard laboratory and greenhouse (wheat smut) procedure (1) was used to determine the effectiveness of 0.5 per cent, by seed weight, of 126 compounds in controlling stinking smut of wheat. In this method, artificially smutted wheat is dusted with given dosages of the chemicals by adequate rolling, the seed planted in flats and held at 10° C. until emergence. The flats are then removed to 15° to 18° C. where light is available until stem elongation occurs, after which the wheat is matured either in the greenhouse or in the open; counts are then made of the smutted heads. Space and seed available made it impossible to test the number of chemicals used with the relatively large samples recommended in the standard procedure. The following modification was thus made: 2-g. samples of smutted wheat were treated with 0.01 g. of chemical and three randomized rows of 20 seeds each planted for each treatment. The variety Hindi of wheat (*Triticum aestivum* L.) was infected with *Tilletia tritici* (Bjerk.) Wint. race 10 in this work.[3]

REPLICATIONS

Three replicate tests on different days were made with 30 compounds by the slide-germination technique and with 56 compounds by the tomato foliage disease method. Since it has been established that fungi contribute more to the variation obtained in the slide-germination method than do replicates (9), the remaining 569 compounds were tested on four fungi

[3] The authors are indebted to Dr. C. S. Holton, Bureau of Plant Industry, Pullman, Wash., for his kindness in furnishing inoculum of *T. tritici* and seed of Hindi wheat for this investigation as well as for advice on the testing technique.

but not replicated. Due to the large number of compounds examined, it was impossible to test all compounds at the same time by either of these methods. The wheat smut tests were not repeated at different times but three replications were made in a single experiment in which all 126 compounds were included. Further, 98 of these compounds were tested by the tomato foliage disease method in order to obtain correlations with the wheat smut results.

COMPOUNDS

The compounds examined covered a wide range of chemical types and included: copper, chromium, uranium, and zinc among the metallic and organo-metallic compounds, and acids, alcohols, aldehydes, amines, esters, ethers, chlorine compounds, sulphur compounds, and combinations of these types in the organic field.

EXPRESSION OF RESULTS

Laboratory results are recorded as concentrations or doses necessary to give LD_{50} values (9). Greenhouse results are expressed as per cent disease resulting after a spray with 0.2 per cent concentration of the chemical, where unsprayed plants are considered to have 100 per cent disease (7). Unless otherwise stated, values presented are the means for the three replicate experiments. Correlations were made on the logarithms of these values since this allows equal weight to be given each value when values differing greatly in magnitude are included in the comparison (7). All graphical examples are presented on logarithmic scales for the same reason. Greenhouse results were expressed in a manner which differed from that used with laboratory results for reasons detailed in another article (7), where it has been shown that per cent disease response is comparable in precision to LD_{95} values in the greenhouse. The wheat smut data were recorded as per cent smut on the basis of the amount of smut developing in untreated or check lots as 100 per cent. But since a large number of compounds gave either 0 or 100 per cent disease, and since there was a 3.5-fold variation in the amount of disease in the untreated or check rows, the compounds have been divided into three groups as follows: 0 to 0.5 per cent disease, 0.5 to 30 per cent, and 30 per cent or more, the mid-points of which differed significantly on the basis of replicate variation (13).

STATISTICAL EVALUATION

An attempt has been made to present the results graphically and to give ratios necessary for significant differences so that reliance on formal statistics is not necessary to interpret the conclusions reached. However, a quantitative approach to this problem necessitated the use of statistics in determining the ratios for significance. In order not to interrupt the continuity of thought in the text, a discussion of statistical methods used follows while the basic statistics have been collected in Table I.

The commonly used indices of relation between factors when the values expressed differ in magnitude from item to item are the correlation coefficient r, the regression coefficient b, and, from another viewpoint, the standard error of estimate $Sy \cdot x$ (10, pp. 95–141). The correlation coefficient is a pure number which may vary from unity, when there is a perfect positive correlation, through zero when there is no correlation, to minus unity when there is a perfect negative correlation. It has the disadvantage that intermediate values do not directly express the proportion of variation for which it accounts (11, p. 126). Thus a correlation of ± 0.30 leaves a residual variation of the dependent variate not of 0.70 but of 0.95 while a correlation coefficient of ± 0.95 still leaves a residual variation of 0.30.

The regression coefficient is the slope of the line giving the amount of variation in the dependent variate predictable from a unit increase of the independent variate. It is expressed in terms of the units used in calculating it, and as such is a direct estimate of the proportion of one variate accounted for by the other. When, as in the present work, the dependent variate must be arbitrarily selected, two regression lines exist since each variate may, in turn, be chosen as the dependent one. As the present work relies heavily on graphical presentation of results, only the mean of these two regressions gives a correct representation of the correlation.

The standard error of estimate will be the most satisfactory measure for comparisons in a majority of cases for this presentation for the following reasons: it is a direct measure of the variability unaccounted for by the correlation between two factors; it is independent of the range of values considered and of the slope of the regression line; and it may be expressed in the units measured. However, there is no justification for using $Sy \cdot x$ unless it has been shown that r is significant. When r values have been obtained, a simple way of getting $Sy \cdot x$ is: $Sy \cdot x = Sy\sqrt{1-r^2}$ (11, p. 123). Figures 1 to 5 are presented on the same scale so that the standard error of estimate may be visually compared. Graphically, $Sy \cdot x$ is the vertical distance from the solid to the dotted lines in these figures. Ratios necessary for significant differences have been calculated by the relation that, for normally distributed values, $1.96 \times Sy \cdot x$ will be exceeded only once in 20 times (11, p. 124). This is, of course, large sample rather than small sample theory but it has been used only in cases where there were 30 or more paired comparisons made. Ratios appear since correlations are conducted in logarithms and the antilogarithm of the necessary difference is equivalent to a ratio difference of arithmic values (7). The expected range of this ratio was determined, in order to give an indication of the preciseness of the ratio, from the standard deviation of a standard deviation $(\sigma/\sqrt{2N})$ (11, p. 251). This value was added to and subtracted from the determined standard deviation and ratios for significant differences calculated.

Example: In Table I A it will be noted that the intraclass correlation for replicates in the laboratory had an $Sy \cdot x$ value of 0.195. $1.96 \times 0.195 = 0.382$ = difference in logarithms exceeded by only one of 20 replicated comparisons. Antilog. $0.382 = 2.4$, the ratio necessary for significant differences between replicates of the mean LD50 values of four fungi. The range of this ratio was determined as follows: $(0.195/\sqrt{2 \times 58}) = 0.018$, where there were 58 degrees of freedom used in determining $Sy \cdot x$. $0.018 + 0.195 = 0.213$; $0.213 \times 1.96 = 0.418$; antilog. of 0.418 is 2.6; 2.6 is 0.2 greater than the 2.4 ratio specified above.

Where several estimates of a correlation existed, as in the case of correlation of different replicates in the laboratory, the r values were tested for homogeneity through Fisher's Z transformation (3, p. 191). These r values were not obtained from independent data but the homogeneity test was used only as a limitation to the values on which an intraclass correlation could be run. When they were homogeneous, the intraclass correlation (3, pp. 204–231) was used as providing the best available estimate of the true correlation. The $Sy \cdot x$ values thus obtained were used in determining the ratios for a significance given in the text.

RESULTS

As a preliminary to the correlation of laboratory and greenhouse results, it was necessary to determine the correlations existing between replicate tests and different fungi in the laboratory and between replicate tests and different diseases in the greenhouse.

LABORATORY REPLICATES

The reproducibility of replicate test results with the slide-germination method is presented graphically in Figure 1 A, where the averages of the logarithms of the LD50 values for the four test fungi of 30 compounds for one replicate have been plotted against those for another replicate. It is obvious that replicate tests in the laboratory with the slide-germination method are highly reproducible for a biological method. Correlations for all possible comparisons are given in Table I A. The ratio necessary for significant differences between arithmic LD50 values (mean of values for four fungi obtained in a single test) for compounds was 2.4 ± 0.2. This is not to say that such a difference will indicate compounds that are better for all four fungi but that mean arithmic values for these four fungi will differ in replicate tests by chance alone as much as 2.4-fold approximately once in 20 times. This value is for comparisons of dissimilar or heterogeneous compounds and it has been shown elsewhere (8) that, with this method, smaller differences are necessary for significance with like than unlike compounds.

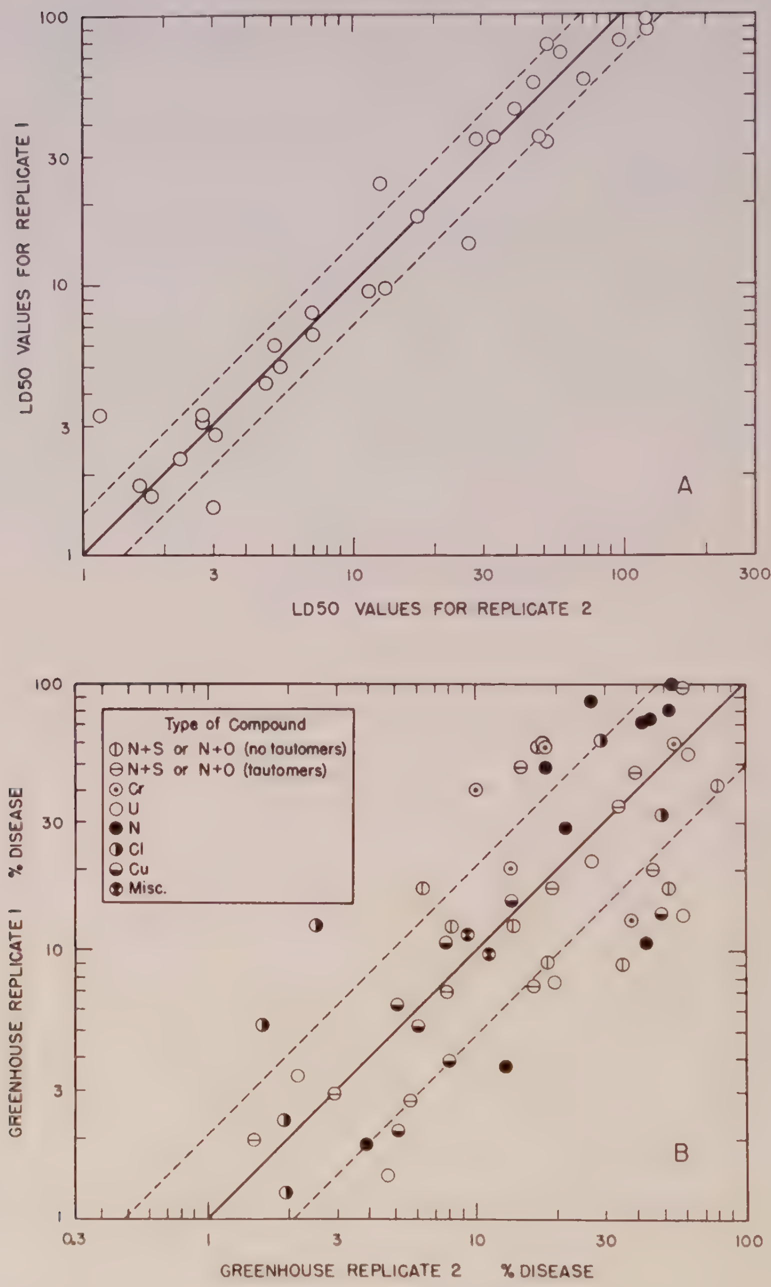

FIGURE 1. Correlation of replicate tests. A. Slide-germination method. LD50 values (mean for four fungi) ($r = +0.982$, $Sy \cdot x = 0.150$). Types of compounds not designated. B. Tomato foliage disease method. Per cent disease values (mean for three diseases) following sprays with 0.2 per cent concentrations of various chemicals ($r = +0.752$, $Sy \cdot x = 0.348$).

TABLE I

CORRELATION COEFFICIENTS r, STANDARD ERRORS OF ESTIMATE $Sy \cdot x$, RATIOS NECESSARY FOR SIGNIFICANT DIFFERENCES BETWEEN COMPOUNDS AND EXPECTED RANGE OF THESE RATIOS

Correlation	r*	$Sy \cdot x$	Significant ratio	Range of ratio
A. Laboratory replicates—LD50 values, mean of 4 fungi				
Replicate 1 vs. Replicate 2 (see Fig. 1 A)	+0.982	0.150		
" 1 " " 3	+0.959	0.221		
" 2 " " 3	+0.971	0.187		
Intraclass	+0.970	0.195	2.4	2.2–2.6
B. Greenhouse replicates—per cent disease, mean of 3 diseases				
Replicate 1 vs. Replicate 2 (see Fig. 1 B)	+0.752	0.348		
" 1 " " 3	+0.626	0.364		
" 2 " " 3	+0.671	0.382		
Intraclass	+0.683	0.373	5.4	4.8–6.0
C. Laboratory fungi—LD50 values, mean of 3 replicates				
Pairs of fungi				
Sclerotinia vs. *Glomerella* (see Fig. 2 A)	+0.777	0.562		
" " *Alternaria*	+0.637	0.638		
" " *Macrosporium*	+0.835	0.456		
Alternaria " *Glomerella*	+0.864	0.416		
" " *Macrosporium*	+0.671	0.614		
Glomerella " "	+0.679	0.656		
Intraclass	+0.740	0.593	15	12–18
Correlation of means				
Sclerotinia and *Alternaria* vs. *Glomerella* and *Macrosporium*	+0.886	0.306		
Sclerotinia and *Glomerella* vs. *Alternaria* and *Macrosporium*	+0.889	0.297		
Multiple regression				
Sclerotinia, *Alternaria*, and *Glomerella* vs. *Macrosporium* (see Fig. 2 B)	+0.888	0.381		
Alternaria, *Glomerella*, and *Macrosporium* vs. *Sclerotinia*	+0.875	0.391		
Theoretical for 4 fungi**	+0.884	0.365		
Average for 5 above values: av. $Sy \cdot x = \sqrt{\frac{\Sigma(Sy \cdot x)^2}{N}}$		0.351	4.9	4.0–6.0
D. Greenhouse diseases—per cent disease, mean of 3 replicates				
Late Blight vs. Septoria Leaf Spot (see Fig. 3 A)	+0.473	0.587		
Late Blight vs. Early Blight	+0.318	0.640		
Early Blight vs. Septoria Leaf Spot	+0.391	0.497		
Intraclass	+0.397	0.532	11	9–13

* All r values presented are significant so that a positive correlation exists in all cases.

** Based on intraclass r for pairs of fungi. Calculated through coefficient of alienation (11).

GREENHOUSE REPLICATES

A similar comparison of replicate variation by the greenhouse tomato foliage disease method is given in Figure 1 B, where the mean per cent disease value for three foliage diseases of 56 compounds obtained in one replicate is compared with that for another replicate. By the widths of standard error zones (distance from solid to dotted lines) it will be seen that replicate values obtained in the greenhouse were less reproducible than those obtained in the laboratory[4]; see also Table I B and compare with Table I A. This has been shown qualitatively in another paper (7) by use of the analysis of variance. The method as used would necessitate a 5.4 ± 0.6 between the per cent disease responses (mean for three tomato foliage diseases) of two compounds for significance. Strictly speaking, in determining replicate correlation, the mean of three greenhouse diseases was used, while in the laboratory the mean value for four fungi was used. By extrapolation[5] a ratio of 4.3 ± 0.4 would have been necessary for significance had the mean of four foliage diseases been used in determining the reproducibility of replicates. This is still much higher than the ratio of 2.4 ± 0.2 necessary for significance with the mean LD50 values for four fungi in the laboratory. It is to be expected that the greenhouse results, in this case, would be less precise than those of the laboratory since the plant variable is present in greenhouse tests. In addition, single tomato plants were used for each disease and replicate and only one dosage was tested, while the LD50 obtained in the laboratory is estimated from at least three finite per cent responses at different dosages. True differences in the order of rating of a series of compounds by different fungi or diseases will add to this replicate variation and necessitate larger ratios for significant differences in determining from the rating on one compound or disease what will be the order of rating on another compound or disease.

LABORATORY FUNGI

It has been shown qualitatively that compounds are not equally toxic to all fungi nor are they rated in the same order by the different fungi (9). However, the magnitude of the divergence in rating to be expected with

[4] The variances $(Sy \cdot x)^2$ for greenhouse replicates were homogeneous for all tests by Bartlett's χ^2 test (10, p. 207), as were those of the laboratory but the laboratory and greenhouse variances were not homogeneous when put together. Thus the reproducibility of results in the greenhouse was significantly lower than in the laboratory.

[5] Since the variance is inversely proportional to the number of items making up the mean (3, p. 112), the variance expected with mean of four diseases would be

$$\frac{3 \times \text{variance for mean of three diseases}}{4} \text{ or } \frac{3 \times (0.373)^2}{4} = 0.104 \text{ and } \sqrt{0.104} = 0.323,$$

the expected $Sy \cdot x$ value if four diseases had been used. $1.96 \times 0.323 = 0.633$. The antilog. of 0.633 is 4.3, the ratio necessary for significant differences.

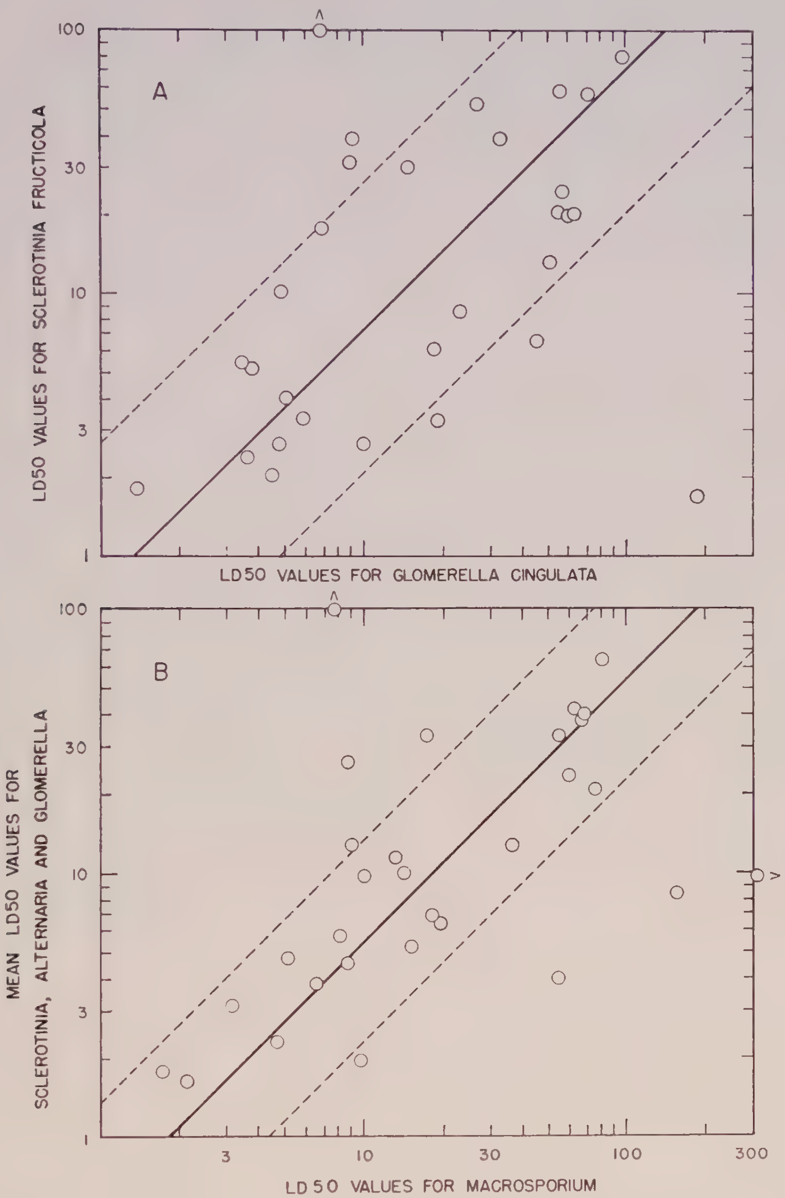

FIGURE 2. Correlation of LD50 values (mean for three replicates) obtained for different fungi by the slide-germination method. A. *Sclerotinia fructicola* versus *Glomerella cingulata* ($r = +0.767$, $Sy \cdot x = 0.562$). B. Mean for *Sclerotinia fructicola*, *Alternaria solani*, and *Glomerella cingulata* versus *Macrosporium sarcinaeforme* ($r = +0.888$, $Sy \cdot x = 0.381$). Symbol $>$ or $\wedge$ indicates greater than.

compounds in general has not previously been shown. Figure 2 A shows the correlation between *Sclerotinia* and *Glomerella* and the values obtained for the other correlations are given in Table I C. Comparison of the $Sy \cdot x$ zone with that illustrated in Figure 1 A shows that the order of rating of compounds varies more with different fungi than would be expected from replicate variation.[6] A ratio of 15 ± 3 is necessary between LD50 values (mean of three replications) of two compounds on one fungus before it can be stated with approximately 20 to 1 odds that the more toxic will also be more toxic to another fungus. This is not due to inherent variation of the method but rather to the fact that dissimilar compounds are rated differently by different fungi so that while 19 compounds out of 20 may not be expected to differ in their relative ratings by more than 15-fold on two different fungi, 1 compound in 20 will be expected to differ by 15-fold or more. Thus to allow any certainty of prediction on the behavior of a new fungus this ratio must be exceeded and even then 1 in 20 comparisons may be expected to differ from the prediction. It should be pointed out that all the test fungi were Ascomycetes or Fungi Imperfecti and that the conclusions reached do not necessarily hold if predictions are from toxicity to these fungi to toxicity toward Phycomycetes or Basidiomycetes.

Additional replications increase the precision of the mean and consequently of the comparisons to be made. It has been assumed in the past that this also holds for the inclusion of additional fungi in the testing technique (9). It has not been shown previously how much is gained in predicting toxicity to a new fungus by the use of the mean values for four fungi rather than the value for one fungus. In Figure 2 B is shown the correlation of the mean LD50 values for *Sclerotinia*, *Alternaria*, and *Glomerella*, and the LD50 values for *Macrosporium*.

It is apparent that the correlation is better, i.e., the $Sy \cdot x$ mean zone is narrower, than in Figure 2 A. It is shown in Table I C that the ability to predict order of toxicity toward fungi is similar when the mean value for two fungi is used to predict the mean LD50 value for two other fungi to that when the mean for three fungi is used to predict the LD50 value for another fungus. It is logical to calculate an average ratio for significant differences, which is necessary between mean LD50 values for three fungi, in order to predict order of toxicity to a new fungus. This ratio, calculated for these test fungi, is 4.9 ± 0.9.[7] If two compounds differ 4.9-fold in the mean of the LD50 values obtained for three fungi, it would be expected that the more toxic would also be more toxic to another fungus in approxi-

[6] The $Sy \cdot x$ values obtained in the correlation of fungus with fungus were not homogeneous with those obtained in comparing replicates in the laboratory by Bartlett's χ^2 test.

[7] Calculated from the average standard deviation $Sy \cdot x$ (Table I C). Since there are equal degrees of freedom for each value, $\sqrt{\frac{\Sigma(Sy \cdot x)^2}{N}} = \text{av. } Sy \cdot x = 0.351.$

mately 19 out of 20 such pairs of compounds. Since it has been recommended that four test fungi be used, by extrapolation the ratio for significant differences in predicing order of toxicity to a new fungus from the mean LD50 values for four fungi would be 4.0 ± 0.5.[8] This is a marked decrease from the ratio of 15 necessary for prediction when only one fungus has been examined. Thus the more fungi examined the more precisely predictions can be made as to toxicity of a given compound to fungi in general or to any other specific fungus. The advantage derived from testing ten fungi, where a postulated ratio of 2.5 would be necessary, instead of four fungi, would not ordinarily be worth the additional labor involved, since this postulated gain would not be fully realized as the reproducibility of replicate determinations becomes an increasingly important factor in determining ratios necessary for significance as larger numbers of fungi are included in the testing technique. It is, of course, debatable how well any selection of compounds can represent the results to be expected with compounds in general. This criticism of the foregoing discussion has been answered, at least in part, by the selection of compounds of widely different types for use in this test (see section on Compounds under Methods).

Chemically related compounds. Another approach to this problem is to divide compounds into chemically related groups. LD50 values were available for 387 compounds with the four test fungi. These compounds were divided into chemically related groups and analyses of variance conducted within each group on the logarithms of the LD50 values. Compounds were not included in more than one group and unless specifically stated otherwise, the rest of the molecule consisted only of carbon and hydrogen. The error variances (compound × fungus interaction) are presented in Table II, column 7. From these variances may be calculated the ratios necessary for significant differences between compounds by the t test and converting to antilogarithms for the ratio (8). The ratio for differences in toxicity of two amines would be 2.3 ± 0.3, which is in agreement with the ratio found necessary earlier in the paper for significance when the mean value for four fungi in one replicate was compared with that of another replicate. Since these compounds were tested over a period of several months, they are in a sense replicates with different concentrations of the same chemical, if it can be assumed that they exert toxicity in the same manner. With the group having the greatest error variance,[9] namely the thiuramdisulphides

[8] Extrapolated variances for four fungi from both the $Sy \cdot x$ obtained in the intraclass correlation for two fungi and the average $Sy \cdot x$ when three fungi were compared with one fungus. These variances were averaged and the average standard deviation used in determining the ratio necessary when four fungi are compared with one fungus.

[9] Though the variances presented in Table II, column 7, do not differ greatly in magnitude, Bartlett's χ^2 test shows that they are not homogeneous. This can be shown because of the large number of degrees of freedom behind each value (a total of 387 compounds tested).

TABLE II

RELATIVE SENSITIVITY OF DIFFERENT FUNGI TO VARIOUS TYPES OF ORGANIC COMPOUNDS

Type of compound	Mean LD50 for series on arbitrary arithmic scale				Ratio for sign. diff. of mean*	Variance in logs. (Compound ×Fungus interaction)
	Sclerotinia	*Alternaria*	*Glomerella*	*Macrosporium*		
Amines and heterocyclic nitrogen compounds	1.00	2.09	2.69	1.70	1.17	.065
" " " " " plus**	1.00	2.19	2.34	1.70	1.20	.061
N and S on same carbon atom (taut.)†	1.00	2.04	1.35	1.66	1.52	.117
" " " " " " " (no)†	1.00	1.82	1.26	2.14	1.24	.089
Aldehydes, ketones, esters	1.17	1.15	1.05	1.00	1.54	.110
Ethers	1.48	1.00	1.20	1.23	1.30	.067
Hydroxy compounds	2.95	1.26	1.00	2.89	1.66	.074
Acids	4.57	1.00	1.66	1.20	1.45	.097
Sulphur as –SH, –S–, –S–S–	1.35	2.15	1.00	2.40	1.42	.114
Thiuramdisulphides, dithiocarbamates	1.55	2.57	1.00	5.90	2.53	.147
Chlorine	5.13	1.00	2.51	1.15	1.66	.065
Organic uranium	1.20	1.00	2.95	1.24	1.28	.047
Organic chromium	2.09	1.00	1.35	1.86	1.40	.128

* Between mean values for fungi obtained with the corresponding group of compounds. Calculated by *t* test; dependent on variance and number of compounds in group.

** Containing also –OH or –SH groups or –O– or –S– linkages not on same carbon atom.

† (taut.) Tautomerism, the ability of a compound to exist in two isomeric forms which are readily interconvertible, structurally possible; (no) tautomerism structurally impossible (4, p. 219).

and dithiocarbamates, an approximate ratio of 3.5 would be significant under these conditions. The groupings, tautomeric nitrogen+sulphur compounds; aldehydes, ketones, and esters; sulphur (mercapto and thioether) compounds; and thiuramdisulphides and dithiocarbamates, had higher error variances than did the other subdivisions. These were also among the more heterogeneous chemical subdivisions made.

Data are presented in Table II to show that fungi tend to have the same order of susceptibility to chemically related compounds while the order differs with the various groups of compounds. The mean LD50 value with each fungus for all chemicals in a group was obtained from an average of the logarithms of individual values. The arithmic mean LD50 values were then arranged so that comparisons could be made between groups by the formula: (mean LD50 value for fungus/mean LD50 value of most susceptible fungus) = value reported in Table II. This system brings out clearly the differences between relative sensitivity of fungi since it eliminates the confusion of LD50 values of different magnitudes being reported for each group. Also it arbitrarily adjusts the value for the most susceptible fungus to 1.00, and gives the values for the other fungi on an equivalent basis.

It will be seen (Table II) that *Sclerotinia* was twice as sensitive to amino compounds as was *Alternaria*, while with chlorine compounds *Sclerotinia* was five times more resistant than *Alternaria*, and both differences were significant. Every fungus exhibited extremes, being the most sensitive to at least one group of compounds and the most resistant to another. Horsfall *et al.* (5) contend that the protoplasm of fungus spores is equally sensitive to copper compounds and consequently sensitivity for copper compounds depends only on the relative sizes of the spores of the fungi compared. Table II shows that such a relation is not general and instead relative sensitivity of fungi depends on the nature of the toxicant applied.

This approach provides information as to the active group in a chemical when more than one possible toxic group is present in a compound. Thus the first two classes in Table II behave very similarly in their action on the different test fungi and further, the ratio necessary for significant differences between compounds could not be shown to differ from that given earlier in this paper for replicates. Thus it would be concluded that the amino group was the active toxicant in all these compounds even when hydroxyl or sulphydryl groups or ether or thioether linkages were also present in the same molecule.

GREENHOUSE DISEASES

The correlation of diseases in the greenhouse is a similar correlation to that between fungi in the laboratory which was presented graphically in Figure 2. An example of the correlation between diseases in the greenhouse

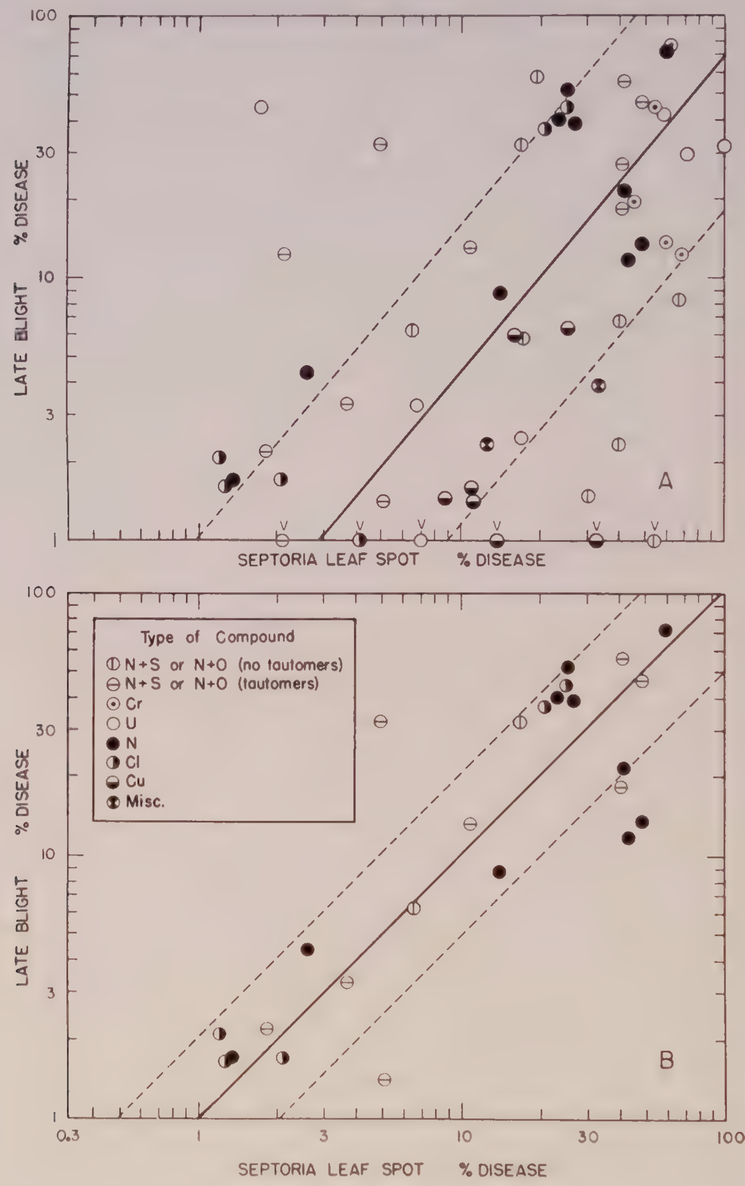

FIGURE 3. Correlation of the per cent disease obtained for Late Blight and Septoria Leaf Spot (mean for three replicates) following sprays with 0.2 per cent of various chemicals. A. With all compounds examined ($r = +0.473$, $Sy \cdot x = 0.587$). B. With a selected group of compounds, omitting copper, chromium, uranium, and miscellaneous compounds ($r = +0.846$, $Sy \cdot x = 0.304$).

appears in Figure 3 A, where Late Blight and Septoria Leaf Spot are compared. From the intraclass correlation, where all three diseases were considered (see Table I D), it was found that a ratio of 11 ± 2.0 was necessary for significant differences. For example, if two compounds, applied at the same dosage, gave 0.5 and 10.0 per cent disease respectively on Late Blight, the chances are that the more effective would also be more effective on Septoria Leaf Spot, though no accurate prediction can be made as to how much more effective it would be on Septoria Leaf Spot. Thus, if a heterogeneous group of compounds is examined, the prediction of effectiveness on a different disease from results with a known disease can only be made within very broad ranges. For instance, given 20 pairs of compounds, one compound of which permitted 9 per cent Late Blight and the other 99 per cent, it would be expected that the order of toxicity of one of these pairs would be reversed on Septoria Leaf Spot. It has been shown earlier in this paper that a similar situation exists when one fungus is correlated with another for a heterogeneous group of compounds. The ratio for significant differences of LD_{50} values in the laboratory was found to be 15 ± 3.5 when predictions were to be made from results on one fungus to those on another. Thus, with heterogeneous or dissimilar compounds, on the basis of these data, predictions of per cent disease response can be made about as accurately from one disease to another in the greenhouse as predictions of LD_{50} values from one fungus to another in the laboratory.

If a selection of types of compounds is made, a closer correlation exists as is shown in Figure 3 B. The compounds chosen for this comparison were limited to nitrogen, nitrogen + sulphur on the same carbon atom (whether or not tautomerization could exist), and chlorine compounds while copper, chromium, and uranium compounds were omitted. This is similar to the division into specific chemical groups in the laboratory tests, which was not deemed advisable with the smaller number of compounds available here. With these types of compounds a 4.0 ± 0.7-fold difference in per cent disease on one disease should be sufficient so that the more toxic would also be more effective in controlling another disease of the types examined. The same decrease in ratios necessary for significance is noted here as was found in the laboratory when compounds were divided into related classes.

CORRELATIONS BETWEEN LABORATORY AND GREENHOUSE METHODS

The correlations previously determined are secondary in importance to the correlation of the laboratory with the greenhouse method. In Figure 4 is presented the correlation between the mean values for three greenhouse diseases and the mean values for four laboratory fungi for the 56 compounds examined in the greenhouse. It will be remembered that no fungus is represented in both laboratory and greenhouse, though different strains of *Alternaria solani* were used in both methods. Figure 4 shows

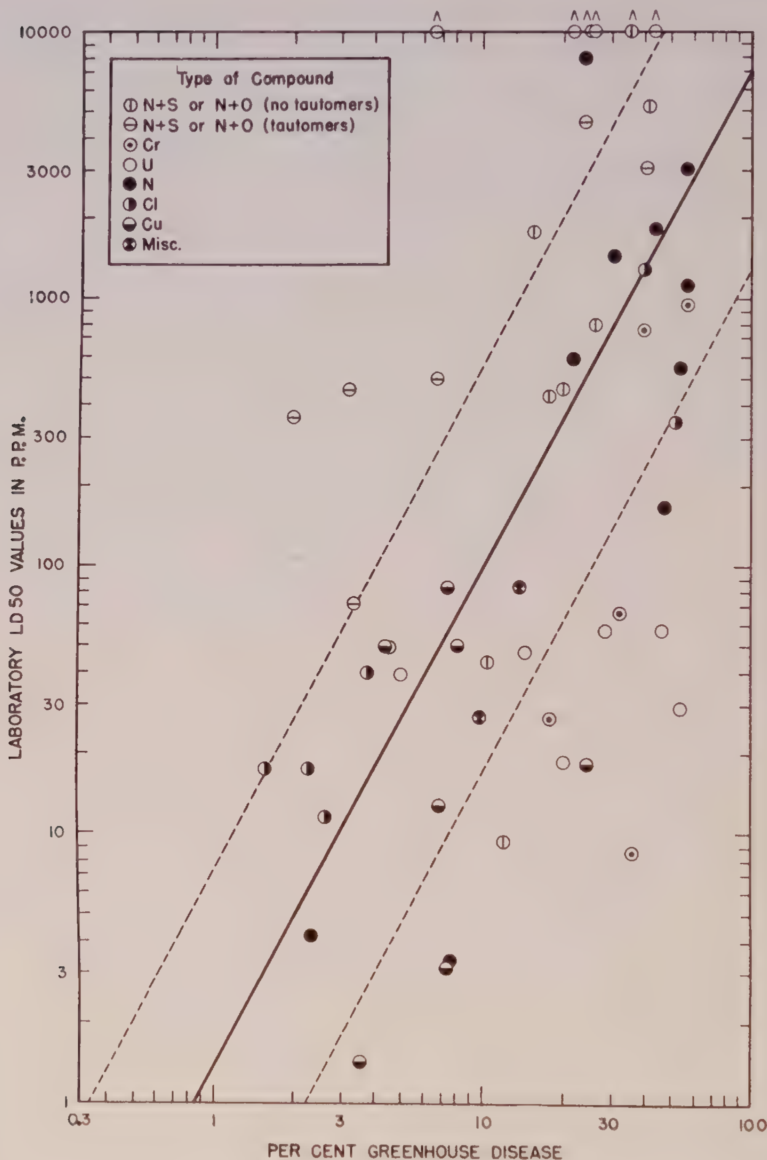

FIGURE 4. Correlation between slide-germination ratings and tomato foliage disease ratings for all compounds examined. Each point represents the mean per cent disease values for three replicates of each of three tomato foliage diseases and the mean LD50 value for four fungi in one experiment ($r = +0.501$, $Sy \cdot x = 0.995$, $Sx \cdot y = 0.398$).

that with the compounds examined, an increase in per cent disease from 1 to 10 per cent (1 logarithmic unit) was accompanied, on the average by an approximate increase in LD50 values from 1 to 100 p.p.m. (2 logarithmic units). Thus, LD50 values change more rapidly than does per cent disease response. Hence a much greater ratio for significance must be obtained for predicting from laboratory to greenhouse than from greenhouse to laboratory. On the basis of the data presented in Figure 4, these ratios would be: with laboratory LD50 values in predicting greenhouse per cent disease values a calculated ratio of 90 with limits of 58 to 210; with greenhouse per cent disease to laboratory LD50 values a ratio of 5.8 ± 1.0. If then, two compounds differ by a ratio of 90 in mean LD50 values based on four test fungi, the most toxic is to be expected to be most effective in controlling disease in the greenhouse as measured by mean per cent disease for three tomato foliage diseases. Or if one compound has given a 5.8-fold greater per cent disease, mean of three diseases, in the greenhouse than another it should also be expected to have a higher LD50 value, mean for four fungi, in the laboratory.

It is instructive to examine the compounds which are not closely correlated in laboratory and greenhouse tests. From Figure 4 it is apparent that the chromium compounds performed poorly in the greenhouse no matter what their laboratory rating was, while the uranium compounds were given approximately the same laboratory rating but differed in their behavior in the greenhouse. It has been shown that soluble chromium and uranium compounds are fungistatic but not fungicidal (6). If the similarity in slope between fungicidal and greenhouse tests (6, 7) can be used to suggest that a fungicidal effect is necessary to control disease in the greenhouse, an explanation of the behavior of the chromium compounds is apparent. Further, it may be postulated that, while the organic uranium compounds did not differ in their fungistatic effectiveness, they may have differed in their fungicidal effectiveness. This would account for their differing behaviors in the greenhouse. Although arsenicals were not tested in the present experiments, it also has been shown that they are potent fungistatic agents and weak fungicidal agents (6), and it is well known that they are only mildly effective in controlling disease in the field. An alternate explanation has been suggested: these compounds may be soluble enough to prevent germination of fungi on glass slides but may not be soluble enough to prevent infection on leaves where a portion of the soluble compound may combine with the leaf or its excretions.

The other group of compounds that gave poor correlation between laboratory and greenhouse results were those compounds where tautomerization (4, p. 219) was possible, containing either nitrogen and sulphur or nitrogen and oxygen attached to the same carbon atom. Some of these compounds were markedly more effective in the greenhouse than would

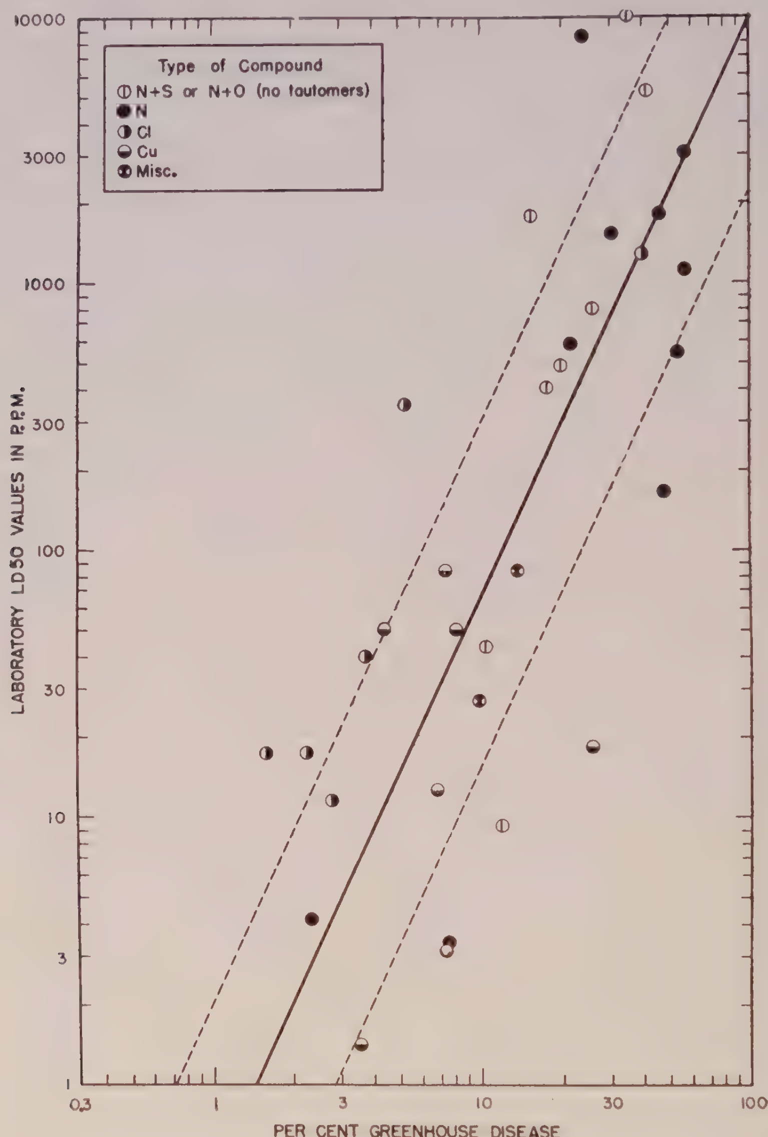

FIGURE 5. Correlation between slide-germination ratings and tomato foliage disease ratings for a selected group of compounds examined, omitting chromium, uranium, and nitrogen tautomers. Each point represents the mean per cent disease values for three replicates of each of three tomato foliage diseases and the mean LD50 value for four fungi in one experiment ($r = +0.772$, $Sy \cdot x = 0.655$, $Sx \cdot y = 0.302$).

have been predicted from the laboratory results. A simple explanation for such a behavior is that, on the plant, these compounds are chemically changed so that the toxic action of the chemical tested in the laboratory is no longer being examined. However, pathogenic *Alternaria solani* spores were found to be germinating on plants which had been sprayed with some of these compounds but no disease lesions developed. Spores were also observed germinating on unsprayed plants in the same experiment but in this case disease lesions did develop in abundance (150 to 300 per plant). Thus it may be that these compounds prevent lesions from forming by some other means than by inhibition of spore germination. Since spore germination is used as a basis for the laboratory tests, toxicity of other types or possible effects on the plant itself could not be measured and hence would not appear from laboratory results. Further experimentation is in progress with the nitrogen tautomers and with the uranium and chromium compounds in an attempt to explain adequately the divergencies that exist in these groups between laboratory and greenhouse ratings. If these three classes of compounds are considered by themselves, there is no significant correlation between laboratory and greenhouse results.

When these three groups of compounds are eliminated the correlation between laboratory and greenhouse tests becomes significantly better as indicated in Figure 5. With the types of compounds in Figure 5, a ratio of 19 (range from 13 to 28) between mean LD50 values (for four fungi) of two compounds in the laboratory would be sufficient to predict order of rating on the basis of mean per cent disease (for three diseases) in the greenhouse. The converse prediction on the same basis, that from greenhouse to laboratory would necessitate a ratio of 3.9 ± 0.6.

The question arises as to whether the correlation between the means of three greenhouse diseases and four laboratory fungi is any poorer than might be expected for the correlation of the mean per cent disease value obtained with three greenhouse diseases with that for three other greenhouse diseases. By extrapolation[10] from the correlation between individual diseases in the greenhouse, a ratio of 4.0 would be necessary between mean per cent disease values (for three diseases) of two compounds to predict the order of toxicity of these two compounds on the mean of three other diseases. Since this ratio is very similar with that found from the correlation in Figure 5, it may be assumed with these types of compounds that prediction from greenhouse to laboratory can be made as accurately as

[10] Following the relation that the variance of a mean is inversely proportional to the number of items that make it up and taking the intraclass correlation $Sy \cdot x$ from Table I D. $\sqrt{\frac{1 \times (0.532)^2}{3}} = Sy \cdot x$ value expected when means of three diseases are compared with means of three other diseases.

from the mean value for three diseases to the mean value for three other diseases in the greenhouse. In other words, with the compounds presented in Figure 5 no additional variation was evident between laboratory and greenhouse results, that could not be accounted for by the variation of diseases in the greenhouse. However, with the chromium, uranium, and nitrogen+sulphur tautomers, which were excluded from Figure 5, there is a real difference between laboratory and greenhouse ratings beyond that which might be expected from correlations between diseases in the greenhouse.

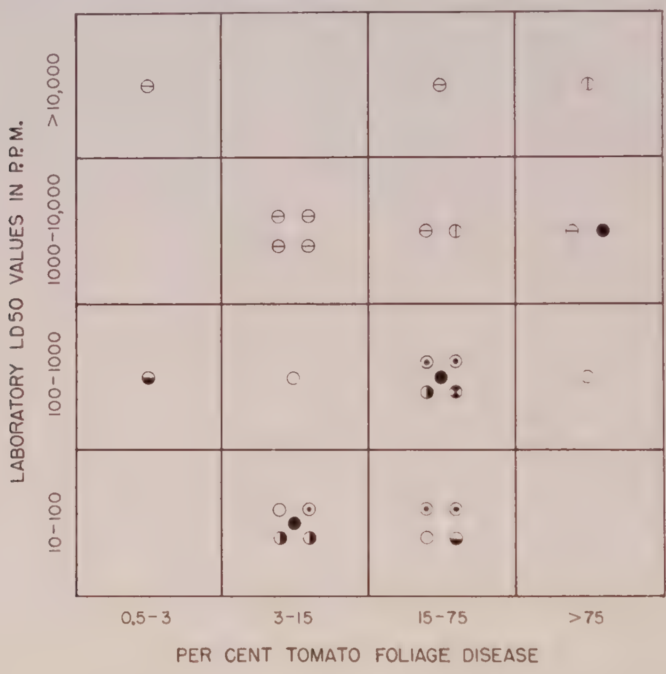

FIGURE 6. Comparison of results with slide-germination and tomato foliage disease methods using the identical fungus, *Alternaria solani* (Maine strain 52). Symbols same as in Figures 1 B, 3, and 4.

The same lack of correlation between laboratory and greenhouse results with the uranium and chromium compounds and nitrogen tautomers is apparent even when the same fungus is tested by both methods. This is shown in Figure 6 where the same fungus, *Alternaria solani* Maine strain 52, was used in both the laboratory and greenhouse. This strain proved to be approximately ten times as resistant in the laboratory as the laboratory strain of *A. solani* and, due to the limited number of spores available, the range of the LD50 values was determined from a dose ratio of 10. It will be seen that even some of the more effective uranium and chromium com-

pounds in the laboratory gave little or no disease control in the greenhouse, while some of the nitrogen tautomers gave very good control in the greenhouse though they showed little toxicity in the laboratory.

Commercial fungicides. Nine commercial fungicides representing copper, sulphur, and organic compounds were compared by the laboratory fungistatic and greenhouse tomato foliage disease techniques. The greenhouse

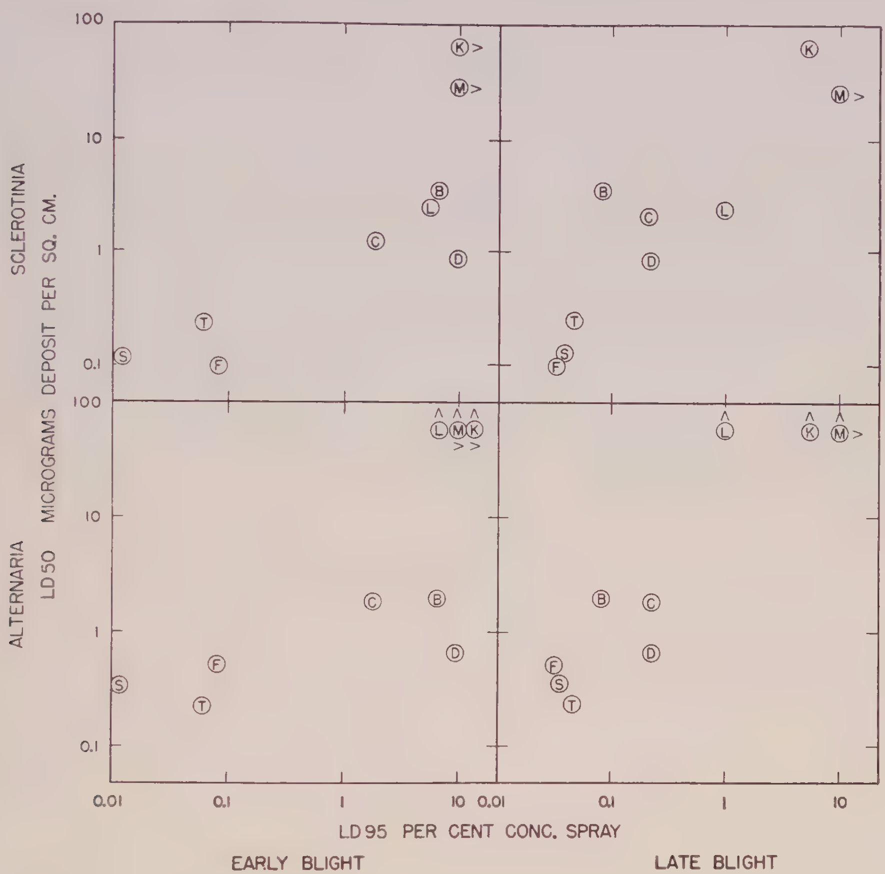

FIGURE 7. Correlation between laboratory LD50 values for *Sclerotinia fructicola* and *Alternaria solani* and greenhouse LD95 values for Early and Late Blights, for various commercial fungicides. B = Bordeaux mixture (conc. as copper sulphate), C = Cuprocide, D = Tennessee Copper "34," K = Kopper's Flotation Sulphur, L = Lime Sulphur (conc. as of 32° Baumé stock), M = Mike Sulphur, F = Fermate, S = Spergon, T = Thiosan.

results expressed as concentration of spray to give 95 per cent control of disease, i.e., LD95, were based on the mean of three tests from data previously obtained (7) on Early and Late Blights. Two tests were performed in the laboratory with *Sclerotinia* and *Alternaria* employing the settling

tower technique. The results are correlated graphically in Figure 7. It will be seen that the correlation approximates that of Figure 5. It is to be noted that in general both fungi and diseases rated the three organic fungicides the most toxic, the three copper compounds intermediate, and the three sulphur compounds the least toxic. It is not to be assumed that these correlations are as representative of compounds in general as previous correlations, as only a few compounds are represented here. Some of these compounds at least were chosen because of promise in both laboratory and greenhouse tests, thus automatically eliminating poorly correlated compounds. They are reported on because they are representative of the fungicides now on the market.

Greenhouse and field. In an experiment in which 26 compounds were tested in the field and in the greenhouse on the same three tomato foliage diseases, compounds fell into the same three classes of effectiveness (excellent, good, and poor) by both tests. In this field experiment the compounds were artificially inoculated with spores within 24 hours after spraying and a period favorable for infection induced by an overhead sprinkler system so that weathering played little part. Thus a close correlation was to be expected as the conditions of the experiment were similar to those of the greenhouse method. If weathering had been a factor, as it is in protecting plants in the field under conditions of natural infection, it is doubted that such a close correlation with laboratory results could have been shown.

WHEAT SMUT

Wheat smut resulting after treatment with 0.5 per cent by seed weight of a series of chemicals is correlated graphically with the laboratory LD50 values, mean for four fungi, obtained with the same chemicals in Figure 8 A and with per cent disease, mean for three diseases, in Figure 8 B. (The per cent disease values were available for only 98 compounds which accounts for the smaller number of comparisons in Figure 8 B.) As explained under Expression of Results, wheat smut data were divided into three classes (0 to 0.5 per cent, 0.5 to 30 per cent, and 30 per cent or more). The comparable divisions made for laboratory and greenhouse data are explained in another paper (13). It is obvious from Figure 8 A that with the compounds examined the correlation between the laboratory and wheat smut data is not high. However, all compounds which controlled wheat smut and yet had laboratory LD50 values larger than 100 p.p.m. were tautomeric nitrogen compounds. Further, three-fifths of the compounds with laboratory LD50 values lower than 10 p.p.m. which allowed wheat smut to develop were chromium compounds though only one-fourth of all compounds that gave LD50 values lower than 10 p.p.m. in this experiment were chromium compounds. These were the same types of compounds

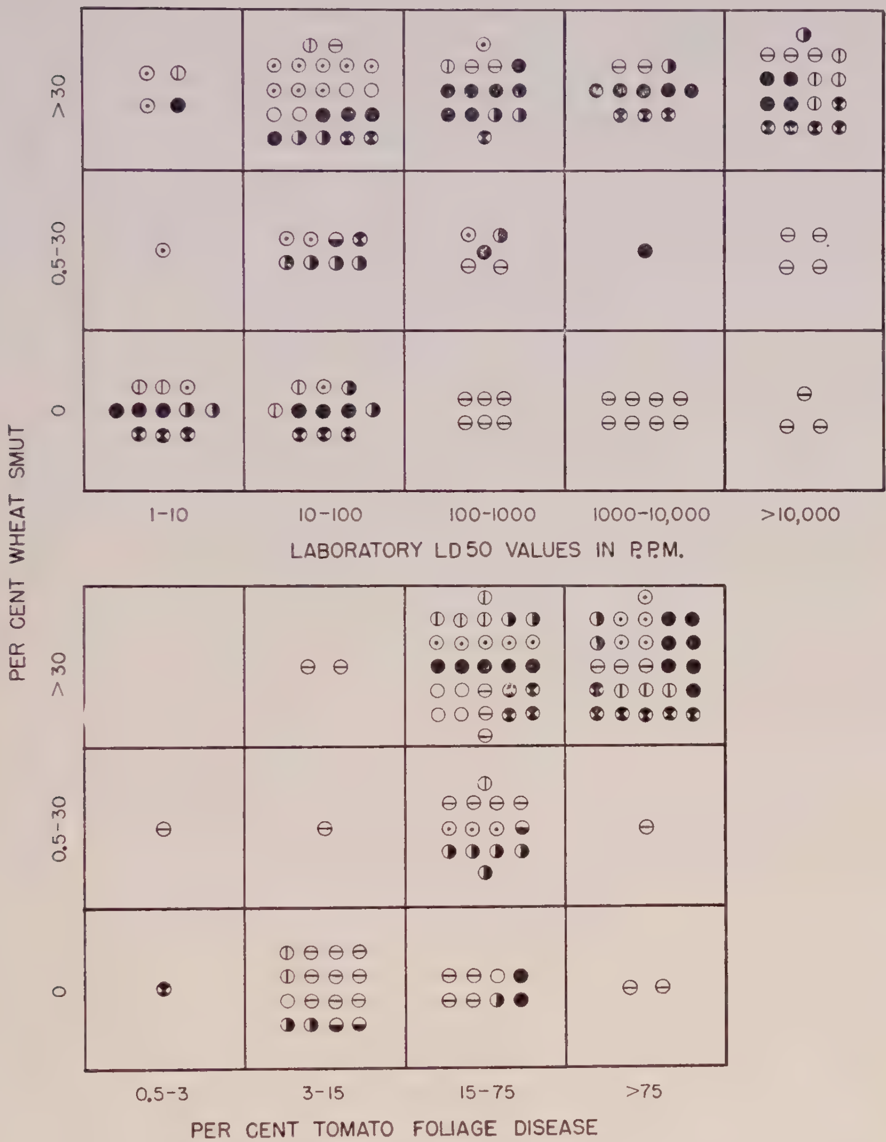

FIGURE 8. Correlations between: A (Upper), wheat smut and slide-germination methods; and B (Lower), wheat smut and tomato foliage disease methods. Symbols same as in Figures 1 B, 3, and 4.

which were giving poor correlation between laboratory and greenhouse results and have been discussed in detail in the previous section. If these compounds are omitted a reasonable correlation between the wheat smut and laboratory methods appears.

A much better correlation exists, when all compounds are included, between the wheat smut and greenhouse methods (Fig. 8 B). The only exceptions, beyond the limits of error of technique, are the tautomeric nitrogen compounds. If it is true that these compounds are chemically changed on foliage and on wheat seed in the soil, it is entirely possible that the change might be influenced in direction, speed, or equilibrium point differently by the diverse conditions of the two tests. While data are not yet available to measure quantitatively this correlation, it is gratifying that two methods which differ so markedly rate compounds in so nearly the same order.

DISCUSSION

It has been shown that, with the majority of compounds examined (these were the nitrogen, nitrogen+sulphur or oxygen where tautomerization was impossible, chlorine, and copper compounds), the correlation between laboratory and greenhouse tests is as good as would be expected from the correlation between diseases in the greenhouse. With these compounds, laboratory fungistatic tests may be expected to give as much information on control of diseases in general as would tests on specific diseases in the greenhouse. Thus, with such compounds, the laboratory and greenhouse tests supplement each other to provide the best estimate of that part of field performance which is dependent on initial toxicity, for diseases in general. Of course, neither of these tests has taken into account resistance to weathering, lack of phytotoxicity or compatability, all of which must be considered in predicting field performance. Compounds of this type which give laboratory LD50 values, mean of four fungi, above 500 p.p.m. are not expected to control disease and do not justify further testing. However, due to variation in effectiveness of the same compound on different diseases, it is impossible to say which compound having an LD50 value of 100 p.p.m. or less will be more effective in control of a given disease, though in general the more toxic compounds in the laboratory will be the more effective. Thus it is necessary to select for field tests not the best compounds, but compounds from the better laboratory group (LD50 values less than 100 p.p.m.) and within this group selection should be largely influenced by other factors such as lack of phytotoxicity, resistance to weathering, and compatability. These conclusions appear to hold equally well in selecting compounds of this type for wheat smut tests.

The chromium and uranium compounds were much more effective in the laboratory than in the greenhouse. Such compounds entail additional

work in that they have to be tested in the greenhouse before they can be discarded as unpromising. Unless greenhouse tests are conducted, however, time and expense might be consumed in useless field tests.

The compounds that present the greatest hazard are those, such as the nitrogen tautomers, which are much more effective in controlling diseases in the greenhouse than laboratory fungistatic tests would indicate. If laboratory tests are relied on with such compounds, promising fungicides may be overlooked. With these nitrogen tautomers it is essential that greenhouse tests be conducted no matter what the laboratory rating may be. Since other types of compounds may exist which behave similarly, in exploratory work on new types of compounds, preliminary greenhouse tests should be conducted to determine roughly the correlation to be expected.

Because the laboratory method is the more precise, it is logical that it should be used when differences are to be shown within a series, for example the straight-chain aliphatic amino compounds; or in control work in examining the relative effectiveness of different batches of the same chemical. With a group of less closely related compounds, for example, copper fungicides or amino nitrogen compounds, it would seem desirable to conduct first a laboratory fungistatic test and then greenhouse tests on all those compounds of which the laboratory LD50 values were less than 100 p.p.m. This, of course, predicates a knowledge of the correlation between laboratory and greenhouse tests for the type of compound being examined.

SUMMARY

1. In order to study the correlations between the laboratory slide-germination and tomato foliage disease methods, LD50 values were determined for 599 compounds using as laboratory test fungi: *Sclerotinia fructicola*, *Alternaria solani*, *Glomerella cingulata*, and *Macrosporium sarcinaeforme*. Also the per cent disease values following sprays with 0.2 per cent concentrations of 154 compounds were ascertained for Early Blight, Late Blight, and Septoria Leaf Spot on tomato, var. Bonny Best. Three replicate determinations were made for 30 compounds by the slide-germination technique and for 56 compounds by the tomato foliage disease technique. Further, the per cent stinking smut on Hindi wheat following treatment by 0.5 per cent by seed weight of 127 compounds was determined.

2. Laboratory fungistatic tests are more nearly reproducible than are tomato foliage disease tests. This is in part due to the addition of the host plant as another variable and to the use of single tomato plants for determining per cent disease.

3. There is more variation in the order of rating dissimilar compounds by different fungi in the laboratory or by different diseases in the greenhouse than is accounted for by replicate variation.

4. The order of rating of compounds on a new fungus can be predicted more precisely from the mean values of three fungi than from any one of the fungi alone.

5. The relative sensitivity of different fungi depends on the chemical nature of the toxicant used. Each of the four test fungi was most sensitive to at least one of the groups of chemicals tested and most resistant to another.

6. When fungi exhibit the same relative order of sensitivity to a compound containing more than one possible toxic group as they do toward another compound containing only one possible toxic group, it is assumed that the toxic group of the former is similar to that of the latter.

7. Among tomato foliage disease tests, a closer correlation exists between diseases when only nitrogen, nitrogen+sulphur on the same carbon atom, and chlorine compounds are included than when the correlation includes also chromium, uranium, and copper compounds.

8. The majority of the compounds examined, the nitrogen, nitrogen +sulphur or oxygen where tautomerization was impossible, chlorine, and copper compounds, gave as close correlation between slide-germination and tomato foliage disease tests as would have been expected in correlating the means of the same number of diseases in the greenhouse.

9. Some of the uranium and chromium compounds were more effective in the laboratory than in the greenhouse. Some of the nitrogen+sulphur or oxygen compounds in which tautomerization could exist were markedly more effective in controlling tomato foliage diseases than would have been predicted from their toxicity in slide-germination tests. With these groups of compounds the same marked differences in rating by the two methods persisted even when the same fungus was used in both tests.

10. Wheat smut results were more highly correlated with tomato foliage disease results than with slide-germination results, though the difference was largely attributable to the same groups that gave poor correlation between slide-germination and tomato foliage disease results.

11. In tests of dissimilar compounds, those having LD_{50} values below 100 p.p.m. in the initial slide-germination method should be given greenhouse disease tests before a selection of compounds is made for field experimentation. In the case of the nitrogen tautomers, greenhouse tests must be conducted on all compounds regardless of their slide-germination rating. Since the slide-germination method has the greatest precision, it will provide the most information in control work where the toxicity of several lots of the same chemical is being investigated.

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A SYSTEM FOR CLASSIFYING EFFECTIVENESS OF FUNGICIDES IN EXPLORATORY TESTS

R. H. Wellman[1] and S. E. A. McCallan

In preliminary fungicide tests it is often neither feasible nor desirable to determine LD values, per cent disease, or phytotoxic effect closely enough to express them numerically. The previous paper (7) has shown that dissimilar compounds are rated differently by various methods of testing and hence, in such comparisons, information of wider application can be obtained by preliminary tests with several methods rather than by a precise test with a single method. Precise information is necessary for accurate comparisons but, in exploratory work, selection of promising compounds can proceed rapidly by merely dividing them into classes of effectiveness. A system for such a classification, which has proved useful, is presented.

METHODS

The methods of testing for which class ratings will be shown in Table I are: the slide-germination, tomato foliage disease, wheat smut, and greenhouse phytotoxicity. The first three are different methods of evaluating fungicides and have been described in detail elsewhere (2, 4, 1).

Slide-germination. The fungi used in the slide-germination method were: *Sclerotinia fructicola* (Wint.) Rehm., *Glomerella cingulata* (St.) Sp. & von S., *Alternaria solani* (Ell. & Mart.) Jones & Grout, and *Macrosporium sarcinaeforme* Cav. This method consists in placing drops containing a known concentration of chemical and of spores on glass slides and observing germination after 24 hours. Some compounds were applied by the settling tower technique (6) and others examined by the test tube dilution technique (5).

Tomato foliage disease. The variety Bonny Best of tomato (*Lycopersicon esculentum* Mill.) was used as a host plant and the three foliage diseases were: Early Blight caused by *Alternaria solani*, Late Blight caused by *Phytophthora infestans* (Mont.) de Bary, and Septoria Leaf Spot caused by *Septoria lycopersici* Speg. Single tomato plants are sprayed under standard conditions with the desired concentration of the chemical in a water base, are allowed to dry, atomized with the specified concentration of spores, placed immediately in high humidity chambers and held there 24 hours to allow for infection, removed to the greenhouse until disease appears when lesion counts are made on three comparable leaves on each plant (4).

Wheat smut. Stinking smut or bunt of wheat was produced on the

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variety Hindi of wheat (*Triticum aestivum* L.) by infection with *Tilletia tritici* (Bjerk.) Wint. race 10. Artificially smutted wheat was dusted with given concentrations by seed weight of the chemicals after which the seeds were planted in flats and held at 10° C. until emergence. The flats were then removed to greenhouses, or other places where light was available, and held at 15° to 18° C. until stem elongation occurred. From this time to

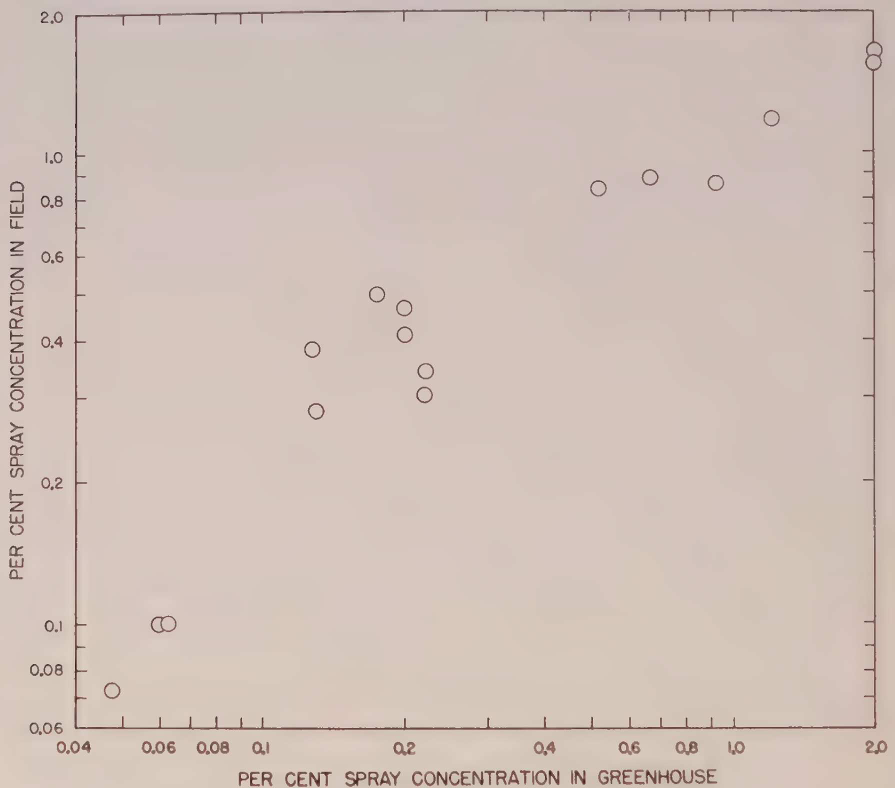

FIGURE 1. Correlation between foliage injury in greenhouse and field. Highest concentrations of spray causing no injury, i.e., the threshold of injury, for a group of organic compounds. Greenhouse results based on average response from bean, buckwheat, and tobacco; field results average response from bean, carrot, cucumber, potato, and tomato.

maturity the plants were held under normal growing conditions and, at maturity, counts were made of the smutted heads (1).

Greenhouse phytotoxicity. The greenhouse phytotoxic effect was determined by applying single doses or a series of doses of a chemical to bean (*Phaseolus vulgaris* L., var. Bountiful), buckwheat (*Fagopyrum esculentum* Gaertn.), and tobacco (*Nicotiana tabacum* L., var. Turkish) by the same

technique used for applying chemicals in the tomato foliage disease method (4). After spraying, the plants are held a week in the greenhouse and records of injury taken. If only a single dose has been applied, the injury is grouped into five grades of severity from none to plant dying (see column 8, Table I). If a series of doses have been applied, a recording is made of threshold of injury. This is the highest dose, usually recorded as the concentration of the chemical in the spray, which produced no observable injury. Such a method is suited to evaluate phytotoxicity which develops directly from the chemical applied but not for evaluating such injury as results from the copper compounds which is caused by liberation of ionic copper over a period of time after spraying (8). Figure 1 shows that with a series of organic compounds there was a close correlation between greenhouse and field phytotoxic effect even though the species of plants sprayed were not the same in both cases.

EXPLANATION OF CLASSIFICATION

If two alternate ways of expressing results of a single method exist, any adequate classification will be one that is so adjusted that the greater part of the compounds tested will fall into the same class by either manner of taking results. Such a classification is presented in Table I, where it will be noted that results for each of the four methods of testing compounds may be taken in either of two ways.

The adjustment was made by recording results in both ways with a series of compounds, then setting the limits for both so that they coincide. In the slide-germination method these consist of the two techniques of applying the chemical; test tube dilution (5) equivalents appear in column 2, and settling tower (6) equivalents appear in column 3. The two ways of recording tomato foliage disease results are explained in detail elsewhere (4). The LD_{95} is the dose or concentration of spray giving 95 per cent control of disease. No "E" grouping could be made for either subdivision (see Table I, columns 4 and 5) in the tomato disease method because the "D" classification had included compounds giving no control and it is impractical from both the mechanics of spraying and phytotoxic effect involved to spray suspensions more concentrated than 10 per cent. The first manner of recording wheat smut results (column 6) has been described in a previous paper (7) and both this and the second manner are comparable to those of tomato foliage diseases. In column 6 no value is given for the "A" or "AA" classes, because when 0.5 per cent by seed weight of the chemical has been used, distinctions cannot be made above the "B" class. With the small lots of wheat necessary in exploratory tests (7), it is impractical to use lower concentrations than 0.5 per cent. Thus if distinctions are to be made between "AA," "A," and "B" classes, such compounds will have to be examined separately using larger amounts of wheat

TABLE I

CLASSIFICATION OF EFFECTIVENESS OF FUNGICIDES IN EXPLORATORY TESTS

Class	Laboratory slide-germination		Greenhouse tomato foliage disease		Wheat smut		Greenhouse phytotoxicity	
	Test tube dilution LD_{50} in p.p.m.	Settling tower LD_{50} in micrograms deposit per sq. cm.	Per cent disease at 0.2% spray	LD_{95} in per cent spray	Per cent disease at 0.5% chemical	LD_{95} in per cent chemical	Injury at 1% spray	Threshold of injury as % spray
AA	<1	<0.1	0 – 0.5	<0.01		<0.02		>5
A	1– 10	0.1– 1	0.5– 3.0	0.01– 0.1	(see text)	0.02– 0.08	None	1 –5
B	10– 100	1 – 10	3 –15	0.1 – 1.0	0 – 0.5	0.08– 0.4	Slight	0.25–1.0
C	100– 1000	10 – 100	15 –75	1 –10	0.5–30	0.4– 2.0	Moderate	0.05–0.25
D	1000–10,000	100 –1000	>75	>10	>30	2 – 10	Severe	0.01–0.05
E	>10,000	>1000				>10	Plant dying	<0.01

which will allow for the testing of the chemical at lower concentrations (1).

It is obvious that any given compound will not necessarily achieve the same rating in slide-germination, tomato foliage disease, and wheat smut methods (7). However, the classes presented for these three methods have been arranged, by means of the correlation presented in Figures 5 and 7 of the previous paper (7), so that the rating by one method will also be the most likely rating for the other two methods when compounds in general are considered. Thus the classes set up are somewhat interrelated and cannot be regarded as entirely arbitrary. Also, the more fungicidal a compound is the more likely it is to have phytotoxic effect. For example, with a series of organic compounds, only 22 out of 141 compounds that had "E" slide-germination rating gave any injury in phytotoxic tests at 1 per cent concentrations, while 92 out of 111 compounds having a "B" or better slide-germination rating were also phytotoxic in 1 per cent concentrations. It is advantageous, however, to have the same classes connote desirability with all methods, and as lack of phytotoxicity is desirable in a foliage fungicide it should be designated by an "A" rating. Increasing phytotoxicity thus proceeds from "AA" to "E" classes while increasing fungicidal action proceeds from "E" to "A" classes. A large number of compounds covering all fungicidal classes were rated by the phytotoxic method so that a phytotoxic range was determined which was comparable to that for fungicidal effect.

It is intended that as other methods are developed, similar classes be made for the results obtained. Sufficient compounds have been tested so that examples of all the classes from "A" to "E" given in Table I are known. A special class, "AA," has been created for compounds that give exceptional performance by any of the different methods. An example of such a compound would be silver nitrate on *Sclerotinia fructicola* and *Alternaria solani* by the slide-germination method (3). Bordeaux mixture, on a copper content basis, has an "A" rating on *S. fructicola* and *A. solani* by the slide-germination method, while by the tomato foliage disease it has an "A" rating on Late Blight and a "C" rating on Early Blight. Ceresan has an "A" rating by the wheat smut method. It is essential, in reporting classes of effectiveness, that the fungus or disease used be specified as well as the method of determination.

SUMMARY

In preliminary tests, where precise determinations are unnecessary, fungicides are classified as AA, A, B, C, D, or E, by various methods, depending on their effectiveness. The class limits for four methods are presented in a table. In the laboratory fungistatic test an AA compound has an LD_{50} value of less than 1 p.p.m.; A, 1 to 10; B, 10 to 100, etc. Tomato foliage disease results are classified either as per cent disease at

a spray concentration of 0.2 per cent, or as LD95 values expressed in per cent. Likewise, wheat smut results are classified as per cent disease with 0.5 per cent chemical, or LD95 values in per cent. Phytotoxicity toward bean, buckwheat, or tobacco is classified as to extent of injury following a 1 per cent spray, i.e., A—no injury, B—slight, C—moderate, etc.; or as to the highest concentrations causing no injury.

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EMPIRICAL PROBIT WEIGHTS FOR DOSAGE-RESPONSE CURVES OF GREENHOUSE TOMATO FOLIAGE DISEASES

S. E. A. McCallan

In an earlier paper the development of a greenhouse method of evaluating fungicides by means of tomato foliage diseases was described in detail (4). The number of lesions developing on treated plants is expressed as per cent of the number of lesions on the control plants and hence the per cent disease or conversely the per cent disease control may be obtained. When the per cent disease is plotted against the spray dosage on logarithmic probability paper a straight line was obtained in most cases. However, it was found that when the per cent response was expressed as probits (1), the LD50 instead of being the most precise point was actually less precise than the LD95, i.e. 95 per cent disease control, or 5 per cent disease.

In the case of precise comparisons it is customary to convert the data to probits and logarithms of dose and calculate the best straight line of dosage-response. However, for such a computation it is essential to have the weights for the different probits. Even when the LD values are obtained graphically (6) it is desirable to have the approximate weights. It is the purpose of this paper to evaluate the precision, and hence weights, associated with different probit levels of response in greenhouse tomato foliage diseases.

The weighting coefficient, z^2/pq, as given by Bliss (1) and also by Gaddum (2) is based on the assumption that the logarithms of the individual lethal doses are normally distributed. In the special case of the greenhouse tomato foliage diseases, and presumably also other diseases in which the number of infections is expressed as per cent of the control, there is at present no theoretical explanation for the distribution of the lesions; hence the weighting coefficients cannot be obtained by theoretical calculations. The problem is further complicated by the high variability between replicate plants.

DETERMINATION OF WEIGHTING COEFFICIENTS

It appeared, in continuation of the earlier studies (4), that a solution might be obtained empirically from actual data on replicate plants since the weight of an observation is equal to the reciprocal of the variance. Accordingly, all the available data from this laboratory on sprayed replicate plants infected with Early Blight or Late Blight were obtained. The number of check plants per test was 2, 3, 4, or 6, and the mean number of check lesions for the different tests varied from approximately 20 to 700.

There were thus available 293 pairs of replicate plants infected with Early Blight and 138 pairs infected with Late Blight, giving a total of 431 pairs of replicate plants. The response in per cent disease per plant varied from 0 to over 100. In a few cases both replicate plants had the same number of lesions, which of course gives the same per cent disease and zero variance for replicate plants, a number unusable for this study. These limited cases were adjusted by assuming that in the one replicate there was one-fourth more of a lesion and in the other one-fourth less of a lesion. The ultimate case where both replicates showed 0 lesions was not considered.

The variances in per cent disease from the various paired replicates were then calculated, and the reciprocal of the variance, or weight obtained. The weight was plotted against the mean per cent disease for the two replicates, as originally obtained and in several transformations. It was found that when the logarithm of the weight was plotted against the logarithm of the mean per cent disease a linear relation existed as shown in Figure 1. It will be noted that the logarithms of the weights for different tests at a given mean per cent disease are not normally distributed. The distribution is somewhat asymmetrical with positive skewness; the asymmetry is more pronounced when the weights are plotted in the original numbers. In order to avoid negative values in the computations, the weights were multiplied by 10,000 and the mean per cent disease by 10, before converting to logarithms, and the values below are so expressed. The regression equation E (7, p. 104) was calculated to be $5.9574 - 1.4312X$ and the standard error of estimate, $S_{y.x}$, was 0.9159. The standard error of the regression coefficient, S_b, equals 0.0648, which gives a t value of 22.08. Since the DF is 429, a highly significant regression coefficient can be shown.

A comparison was made of the Early and Late Blight data and the regression coefficients, b, were determined to be respectively 1.2717 and 1.4812. Although each regression coefficient was found to be highly significant, no significant difference could be shown between the two, thus indicating parallelism (3, p. 42). At the LD95 value, i.e. 5 per cent disease, the logarithm of the per cent weights ($\times$10,000) was 3.850 for Late Blight and 3.265 for Early Blight. The difference, 0.585, was highly significant; this also may be expressed as the ratio 3.85, thus confirming the earlier studies (4) that the Late Blight method is more precise than the Early Blight. However, since there is no significant difference in the regression coefficients, the relative weights for the two diseases are the same. The data for Early Blight were divided into two groups, the one with check plants having a mean number of lesions less than 200 (mean of all tests 98), the other with the mean checks having greater than 200 lesions (mean of all tests 352). There were 84 pairs of treated plants in the former and 209 in the latter. A comparison of regression coefficients and logarithms of weights at LD95 showed no significant difference in either case. It would be

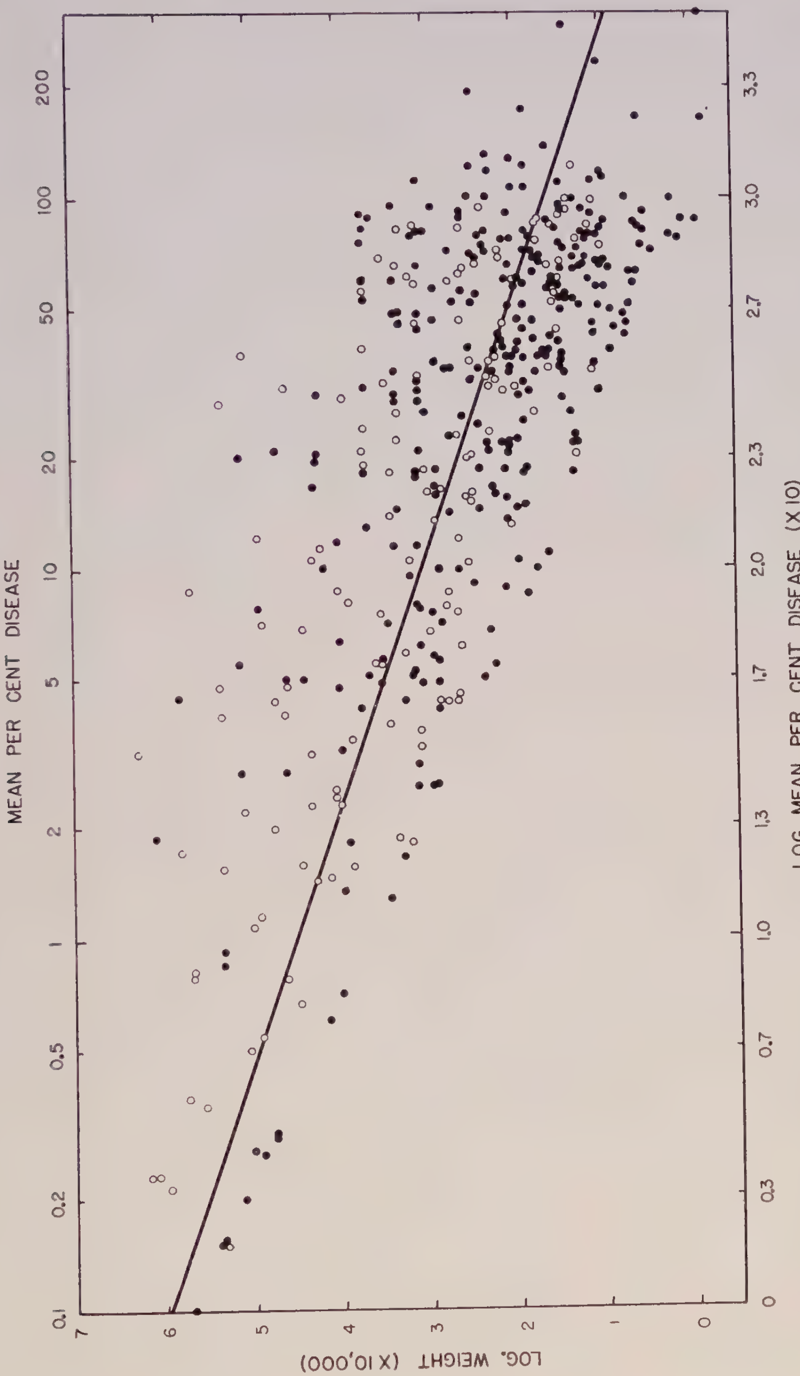

FIGURE 1. Regression of logarithm of weights on logarithm of mean per cent disease. Weights or reciprocal of variance derived from per cent disease on two replicate plants. Early Blight solid circles, Late Blight open circles. $Y = 5.9574 - 1.4312X$.

of interest to know if other greenhouse diseases, or diseases in general under field conditions, would give a regression of logarithm of weights on logarithm of mean per cent disease parallel to that for Early or Late Blights of tomato.

The final probit weights were derived from the per cent weights by multiplying by the appropriate z^2 value as obtained from a table of ordinates of the normal curve (7). This computation has been made and the resulting probit weights are shown in Table I and Figure 2. In order to facilitate the use of logarithmic probability paper, the equivalent per cent disease control for each probit is also given in Table I.

TABLE I

WEIGHTING COEFFICIENTS POR PROBITS IN GREENHOUSE TOMATO FOLIAGE DISEASE DATA

Probit	Equivalent % disease control	Weighting coefficient	Probit	Equivalent % disease control	Weighting coefficient
1.0	99.998	2.4	4.8	57.9	24.3
1.5	99.98	5.8	5.0	50.0	19.8
2.0	99.86	11.4	5.2	42.1	15.4
2.2	99.74	14.8	5.5	30.9	9.7
2.5	99.38	19.7	5.8	21.2	5.4
2.8	98.6	26.4	6.0	15.9	3.5
3.0	97.7	30.2	6.2	11.5	2.1
3.2	96.4	33.6	6.5	6.7	0.85
3.5	93.3	37.2	6.8	3.6	0.30
3.8	88.5	38.4	7.0	2.3	0.14
4.0	84.1	37.7	7.2	1.4	0.059
4.2	78.8	35.6	7.5	0.62	0.014
4.5	69.1	30.8	8.0	0.14	0.00089

The weighting coefficients for probits also could have been determined from the data by converting the per cent disease of replicate plants to probits and calculating the variance and its reciprocal. This was not done because in the first place it would not be possible to use data from plants giving 0 or 100 per cent or more response; secondly, it would be necessary to fit a curved line to the resulting weights or to use an involved transformation giving a straight line. The reverse of the transformation used above will give a straight line; namely, the probit weight is divided by z^2, converted to logarithms, and plotted against the logarithm of the per cent derived from the mean probit. The weights from 12 pairs of replicate determinations were obtained by both methods and no significant difference could be shown between the regression coefficients for the resulting two straight lines. Also, it appears that the distribution of the logarithm of the probit weights likewise is asymmetrical with positive skewness.

It is to be noted in Table I and Figure 2 that the maximum weight, 38.4, is attained at an approximate probit value of 3.8 which is equivalent

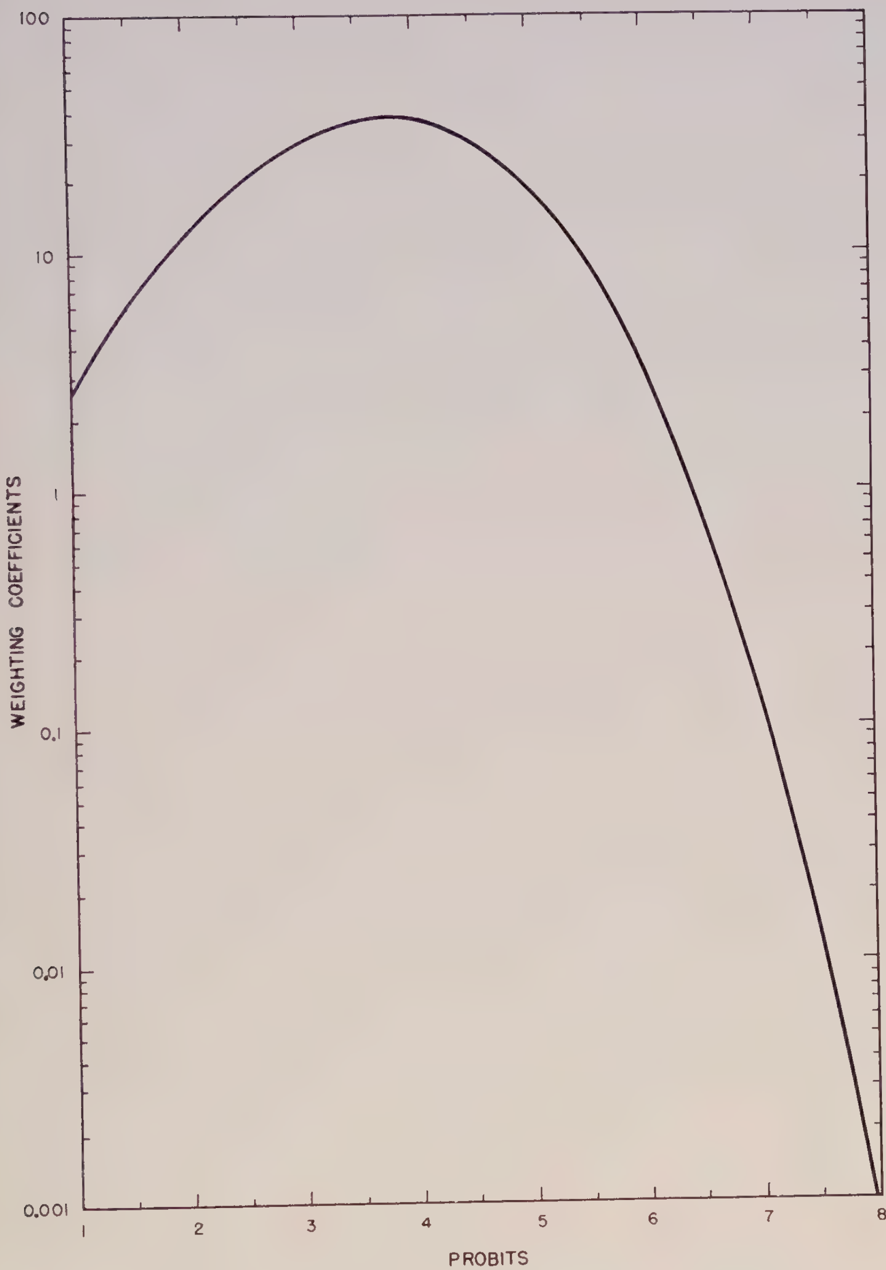

Figure 2. Weighting coefficients for probits in greenhouse tomato foliage disease data.

to 12 per cent disease or the LD88. However, there is little difference in precision within the range LD80 to LD95. The LD50 is only about half as precise as the LD95 and below the LD50 the precision falls rapidly. In the use of this greenhouse method it is suggested that comparisons of dosage for equal response be made at the LD95 level only since the LD95 is in the most precise range, is of most practical interest, and has established usage. If it is desired to obtain the precise LD95 together with its error, the dosage-response curve may be calculated by the orthodox method of Bliss (1), but the above probit weighting coefficients must be substituted. Usually, however, it is desirable to obtain LD95 values from repeated tests and from these to obtain the error term (5) so that the internal error is not necessary. In these more general cases an adequate LD95 may be obtained entirely by graphic methods as described elsewhere (6). Thus the dosage-response curve is drawn on logarithmic probability paper with the most weight being given to the points within the range 80 to 95 per cent disease control. It may be borne in mind that the 50 and 99.4 per cent disease control points have only half the weight of the points within the 80 to 95 per cent range, while the points below 30 and above 99.9 will have less than one-fourth the value and in most cases can be ignored, provided intermediate points are available.

SUMMARY

When the number of greenhouse tomato foliage disease lesions is expressed as per cent of the check, there is a linear relation between probit disease and logarithm of dose; however, orthodox probit weights are not applicable. Probit weighting coefficients were obtained empirically from 431 pairs of replicate tomato plants infected with Early or Late Blight lesions. By the use of the linear regression equation, a highly significant regression coefficient was found between the logarithm of the weight of per cent disease in replicate plants and the logarithm of the mean per cent disease. No difference could be shown between the Early and Late Blight regression coefficients, though the weights for Late Blight were more than three times greater than those for Early Blight. By means of the z^2 transformation, probit weights were obtained and are shown in a figure and table. The maximum weight is approximately at probit 3.8 equivalent to the LD88. There is, however, little difference within the range LD80 to 95, but beyond this range the weights diminish with increasing rapidity. Comparisons of dosage for equal response preferably should be made at the LD95 level.

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HIGH YIELDS OF β-2-TRICHLOROETHYL-D-GLUCOSIDE AND β-2-TRICHLOROETHYLGENTIOBIOSIDE FROM TOBACCO PLANTS TREATED WITH CHLORAL HYDRATE

LAWRENCE P. MILLER

Experiments with a number of species of plants have shown that the presence of considerable quantities of chloral hydrate is tolerated in the nutrient medium and that plants grown under these conditions contain glycosides of trichloroethyl alcohol. In tests with tomato plants (6) it was estimated that the expressed juice from plants grown in a nutrient medium to which chloral hydrate had been added contained as much as one gram of β-trichloroethylgentiobioside per 100 cc. Similarly it was possible to isolate nearly one gram of trichloroethyl glycosides per 100 g. of fresh weight of dandelion leaves (8) from plants grown with chloral hydrate in the nutrient medium. The addition of the large amounts of chloral hydrate necessary to attain such high concentrations of the glycosides within the plants did not result in any marked injurious effect on growth, although the treated plants were somewhat smaller and in some cases showed browning of the edges of the leaves.

In the experiments with tobacco (*Nicotiana tabacum* L. var. Turkish) described in the present paper, even larger amounts of chloral hydrate were used. In the first large series grown to furnish material for the characterization of the glycosides formed, no obvious effect on growth was observed even though the amount of chloral hydrate added to each culture was 70 per cent higher than that previously used with tomato plants, and the expressed juices contained larger quantities of absorbed chemical than had been obtained in the tomato experiments. In later work still larger quantities of chloral hydrate were added to the cultures and tobacco leaves were obtained which contained about 13 per cent on a dry weight basis of a mixture of β-2-trichloroethyl-D-glucoside and β-2-trichloroethylgentiobioside. In these experiments considerable injury was evident but the plants produced viable seed even though most of the plants in this series had been subjected to large amounts of chloral hydrate for most of their growth period from the time the stems began to elongate. The seeds did not contain detectable quantities of the added chemical.

These experiments thus show that it is possible, by treatment with chemicals, so to alter the chemical composition of tobacco leaves as to feature non-naturally occurring substances as major constituents, quantitatively, and that this can be brought about without too marked an adverse effect on the normal development of the plants.

Characterization of the glycosides formed from the absorbed chloral hydrate was carried out with two series of plants. In one series the glyco-

sides were obtained as the crystalline acetates, the leaves yielding both β-2-trichloroethyl-D-glucoside tetraacetate and β-2-trichloroethylgentiobioside heptaacetate and the roots only the β-gentiobioside acetate. In another series, starting with an extract of air-dry leaves, and using a procedure similar to that previously used with dandelion leaves (8), both the glucoside and gentiobioside were obtained directly in crystalline form. This represents the first preparation of crystalline β-2-trichloroethylgentiobioside. Its identity was established through conversion to the acetate and propionate which were found to be identical with the corresponding synthetic compounds (6). Deacetylation of β-2-trichloroethylgentiobioside heptaacetate previously obtained from tomato (6) also yielded the same crystalline gentiobioside.

EXPERIMENTAL

Effect of Chloral Hydrate on the Growth of Tobacco Plants

The main purpose of the experiments on the induced formation of glycosides in various species has been to identify the glycosides formed in the different species studied. In growing the plants an attempt has usually been made to add as much chemical to the nutrient medium as the plants could withstand without too serious injury in order to bring about a high concentration of the induced glycosides and thus facilitate their isolation and characterization. Usually only a few controls were carried along to observe in a general way the effect of the chemical on growth. In view of the indications in the early experiments with tobacco that this species could withstand unusually large quantities of chloral hydrate, it was thought of interest to set up an experiment which would give a little more definite information as to the tolerance of tobacco to chloral hydrate. For this experiment it was decided to use sand cultures supplied with nutrients by the drip culture method since under these conditions better growth is obtained than when static cultures are used.

Small seedling plants in which the stems had not yet started to elongate were used. Three concentrations of chloral hydrate, 0.3 millimol (0.0496 g.), 1.2 millimols, and 4.8 millimols per liter in a nutrient solution recommended by Shive and Robbins (10, 7) were compared with the control solution without chloral hydrate. The plants were arranged in the form of Latin Squares to obviate any differences due to position in the greenhouse. Three blocks of 16 cultures each were used, each row of four plants being made up of one of each of the four treatments. The flow of solution through the cultures was regulated so that about one liter passed through in 24 hours. After about ten days it was evident that all concentrations of chloral hydrate had some retarding effect. The experiment was discontinued after 27 days and Figure 1 shows a typical plant from each lot, three days after the end of the test.

The photograph shows that good growth took place with the solutions containing 0.3 and 1.2 millimols of chloral hydrate per liter and that the plants were not killed when 4.8 millimols (0.749 g.) per liter were present. This latter concentration is comparable to the concentration of nutrient salts in the solution. On continuing the cultures after the addition of chloral hydrate was interrupted these plants showed good recovery and subsequently were used in another experiment, described in a later section of the

FIGURE 1. Tobacco plants grown in sand for 27 days by the drip culture method with chloral hydrate present in nutrient solution. Left to right: 4.8, 1.2, 0.3, and 0.0 millimols of chloral hydrate per liter. Photographed, with saucers added, three days after the end of test.

paper, in which they were subjected to further additions of chloral hydrate but nevertheless grew to maturity and produced viable seed.

Some further data on the extent of the growth effect resulting from absorption of chloral hydrate are given in the succeeding section in which the distribution of the absorbed chemical within the plant is reported.

Distribution of the Absorbed Chemical Within the Plant

The work with other species previously studied had shown that organic chemicals absorbed from the nutrient medium are not evenly distributed throughout the plant but that in some species larger amounts are found in the roots, and in others in the tops. In the course of the isolation of the

glycosides formed in tobacco grown with chloral hydrate present in the nutrient medium, in which the tops were subdivided into stems and leaves,

TABLE I

DISTRIBUTION OF NON-IONIC CHLORINE IN TOBACCO PLANTS TREATED WITH CHLORAL HYDRATE

Plant organ	Position starting at top	Av. wt. per plant, g.		Chlorine content per 100 cc. expressed juice			
				Treated		"Control"	
		Treated	"Control"	Ionic, cc. N	Non-ionic, cc. Molar*	Ionic, cc. N	Non-ionic, cc. Molar*
Leaves	1	0.09	0.09	5.23	1.23	4.23	0.02
	2	0.23	0.41	7.08	1.22	5.60	0.05
	3	0.36	0.73	8.21	1.05	6.53	0.03
	4	0.51	1.19	8.64	1.11	6.88	0.09
	5	0.66	1.37	8.84	1.34	7.03	0.05
	6	0.79	1.65	9.99	1.95	7.10	0.13
	7	0.96	1.75	9.66	2.03	7.21	0.05
	8	1.05	2.02	10.03	2.03	6.96	0.17
	9	1.19	2.17	9.70	2.25	6.60	0.21
	10	1.55	2.49	9.54	2.53	6.25	0.13
	11	1.75	2.34	8.91	2.52	6.86	0.15
	12	2.05	2.36	8.38	2.25	6.63	0.29
	13	2.22	2.56	7.94	2.55	6.38	0.20
	14	2.50	2.50	7.84	2.55	6.63	0.21
	15	2.89	2.79	7.49	2.42	5.78	0.25
	16	2.80	2.93	7.11	2.21	6.13	0.24
	17	2.99	3.05	7.13	2.18	5.79	0.25
	18	2.64	3.30	6.79	2.15	5.39	0.28
	19	2.76	3.53	6.73	2.17	5.60	0.33
	20	2.84	3.40	6.75	2.04	5.39	0.25
	21	2.89	3.30	7.00	1.83	5.46	0.28
	22	2.67	3.64	6.31	1.97	5.13	0.25
	23	2.79	3.68	6.59	1.63	4.65	0.32
	24	2.73	3.94	6.00	1.54	4.99	0.37
	25	2.57	4.00	5.53	1.71	4.73	0.31
	26	3.00	3.86	5.61	1.41	4.14	0.28
	27	2.58	4.50	5.01	1.45	3.63	0.39
	28		4.18			2.98	0.32
	29		4.70			3.01	0.32
	30		4.50			2.50	0.33
	31		4.33			2.63	0.29
	32		4.58			2.49	0.29
Stems	Tip and inflorescence	2.20	12.8	3.78	0.63	3.75	0.04
	Top quarter	14.0	8.1	3.13	0.58	3.86	0.00
	Second quarter	20.1	15.5	1.63	0.65	1.39	0.01
	Third quarter	20.1	13.9	1.25	0.70	0.98	0.06
	Last quarter	14.2	12.6	0.94	0.93	1.31	0.02
Roots		18.3	46.0	0.80	1.15	0.65	0.00

* Calculated for a trichloro-compound.

it was found that the leaves contained much more of the absorbed chemical than the stems. It was thought of interest to determine if the amount present in the leaves varied according to relative position along the stem. Thus, starting with plants which had already made considerable growth before treatment with chloral hydrate was begun, the question arose as to the relative amounts of absorbed chemical which would accumulate in the new leaves formed after addition of chloral hydrate was started as compared to leaves lower down on the stem. For this study the ionic and non-ionic (precipitated by $AgNO_3$ only after previous digestion with alkali) chlorine contents were determined separately for each leaf position, and for four portions of the stems and for the roots. The plants used were started in sand cultures when 8 cm. high and the addition of chloral hydrate was begun when they had reached a height of about 70 cm. Each culture, except for the controls, received 2.5 millimols of chloral hydrate dissolved in 50 cc. of water in each of eight applications over a period of 14 days. Two days after the last application the plants were sampled. Composite samples of leaves were obtained from each leaf position, starting at the tip of the plants. Partially dry leaves at the base of the stems were not sampled. The number of leaves not included averaged 10 per plant in the treated series and 6.5 per plant in the controls. With the smaller leaves about 100 were taken for a sample and as the leaves became larger farther down the stem the number of leaves per sample was decreased until for the largest 4 or 5 were sufficient. The leaves were ground through a food grinder and the analyses made on the expressed juice. The data for the ionic and non-ionic chlorine contents for both treated and control plants as well as figures for the average fresh weights are given in Table I. Ionic chlorine is expressed as cc. N per 100 cc. of expressed juice and non-ionic chlorine as cc. molar (calculated for a trichloro-compound) per 100 cc. It is seen that the stems contained only from one-fourth to one-half of the non-ionic chlorine found in the leaves and that the roots contained a quantity intermediate between that of the stems and the leaves. As to the distribution among the leaves with regard to leaf position, the leaves near the tip and those near the base had a content about half of that found in the leaves in the intermediate positions. To show this more clearly the data for the treated plants are plotted in Figure 2. The maximum amounts of non-ionic chlorine were found in the 10th to the 15th leaves from the tip. These leaves correspond to the first leaves formed after the start of the treatment.

The curve for the ionic chlorine content of the leaves is similar in form to that for non-ionic chlorine except that the highest amounts were found in leaves somewhat nearer the tips of the plant than with non-ionic chlorine. The ionic chlorine content of the roots was relatively very low.

The surprising feature of the data obtained from the control plants was the fact that some non-ionic chlorine was found to be present. Repeated

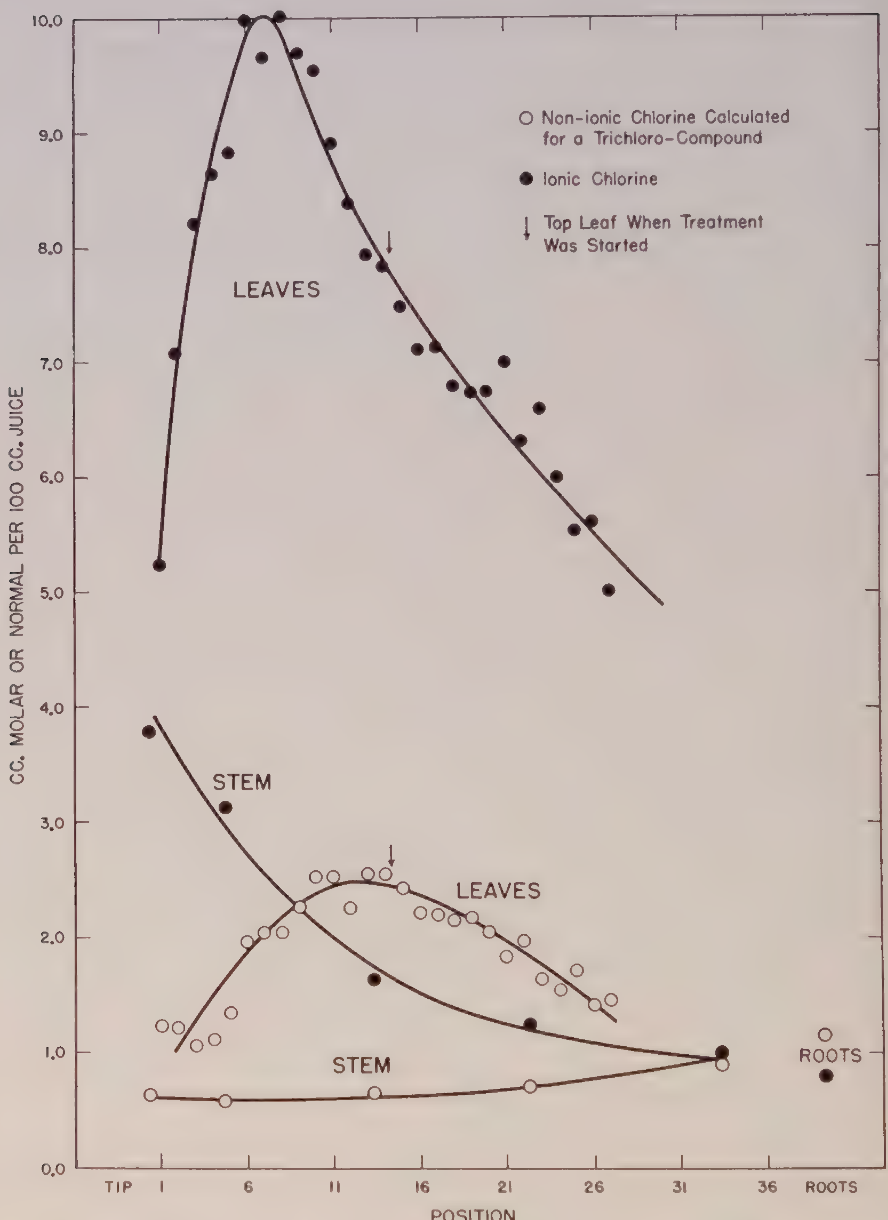

FIGURE 2. Distribution of ionic and non-ionic chlorine in tobacco plants grown with chloral hydrate added to the nutrient medium.

analyses of plants of this and other species which had not absorbed foreign chemicals have always shown such plants to be entirely lacking in non-ionic chlorine as is of course to be expected since higher plants are not known to contain chlorine in organic combinations. Analysis of tobacco leaves from plants in another greenhouse made at the same time as those of the present series again showed no non-ionic chlorine. Contamination from the treatments which were being made in the same greenhouse is therefore indicated. The fact that none of this organically bound chlorine was found in the roots shows that the presence of non-ionic chlorine in the leaves was not the result of inadvertent treatment of some of the control plants by the addition of chloral hydrate to the nutrient medium. The distribution of the chlorine in the leaves with respect to leaf position differs from that of the treated plants since the chlorine is found in large quantities in the lower leaves. It therefore seems that the controls have absorbed the chemical from the air, its presence in the air being due to volatilization from the containers in which the treated plants were growing and perhaps also from the treated plants themselves. That these control leaves actually contained absorbed chloral hydrate was shown by the isolation from some of these leaves of β-2-trichloroethyl-D-glucoside as its crystalline acetate. Details of this isolation are given in a later section of the paper.

The control plants contained slightly less ionic chlorine than the treated plants which suggests that a small amount of the chlorine of the absorbed chloral hydrate may have been changed to the ionic form in the treated plants. The distribution of the ionic chlorine with respect to leaf position is similar to that of the treated plants, the highest amounts being found in the leaves near but not at the tip of the plant and the lower leaves containing only about one-half of this amount.

The plants had an average height of 71 cm. when the addition of chloral hydrate was begun and at sampling time the treated plants were 106 cm. tall while the controls measured 117 cm. Considering the increase in height after the start of treatment with chloral hydrate, the treated plants made about 75 per cent of the growth of the controls. The depressing effect on growth is also shown by the decreased fresh weight of the leaves, stems, and roots as shown in Table I.

CHARACTERIZATION OF THE GLYCOSIDES FORMED

First Series

Sixty tobacco plants about 15 cm. tall which had been grown in good greenhouse soil were transferred to sand cultures in 5-inch clay pots supplied with saucers. Nutrients were furnished by the addition, three times weekly, of 150 cc. of double strength nutrient solution based on a solution recommended by Shive and Robbins (10) and described more fully in a

previous publication (7). After 20 days, when the plants were 38 cm. in height, the addition of chloral hydrate was started at the rate of 0.7 millimol to each of 50 cultures six times weekly. The rate of application was doubled after 18 days and discontinued after each plant had received 15.4 millimols. Two days after the last application the plants were sampled following the procedure previously used with tomato plants treated with chloral hydrate (6). The tops (leaves and stems) and roots were handled separately. Analysis showed the expressed juice from the tops (2835 cc.) to contain non-ionic chlorine equivalent to 1.88 millimols of a trichloro-compound per 100 cc. and that from the roots (800 cc.) 2.56 millimols per 100 cc. Of these amounts only 0.16 and 0.23 millimol from the top and root juice, respectively, were volatile on distillation but after being subjected to hydrolysis by emulsin 1.13 and 1.95 millimols were obtained. These results offer evidence that a major part, at least, of the added chemical is present in the form of β-glycosides.

Isolation of β-2-trichloroethyl-D-glucoside and β-2-trichloroethylgentiobioside from the tops as the crystalline acetates. The procedure followed for obtaining from the expressed juice and additional aqueous extracts a preparation suitable for acetylation and the method of acetylation used were the same as described in a previous publication (6). Acetylation of a portion of the material extracted by acetone from the concentrated lead precipitated juice from the tops gave 9.07 grams of crude acetate melting at 138° to 141° C. (uncorr.). Purification by recrystallization from absolute ethanol was considerably more difficult than with similar preparations previously encountered in work with other species but after repeated recrystallization the product reached a constant melting point of 144.5° to 145.5° C. (corr.). A mixed melting point with synthetic β-2-trichloroethyl-D-glucoside tetraacetate (7) gave no depression. Specific rotation was found to be $[\alpha]_D^{25°} = -28.9°$ (concn., 2.8 g. in 100 cc. $CHCl_3$) as compared to $-29.0°$ for the synthetic compound.

Analysis. Calcd. for β-2-trichloroethyl-D-glucoside tetraacetate, $C_{16}H_{21}O_{10}Cl_3$: Cl, 22.17. Found: Cl, 22.38.

Previous work had shown that in extracting concentrated plant juices with aqueous acetone in a series of extractions the simple glucosides are more easily obtained and therefore predominate in the early extracts while glycosides involving disaccharides are less soluble and are present in larger amounts in subsequent extractions. Therefore a portion of the material obtained by acetone extraction after several previous extractions was acetylated separately to detect the possible presence of an additional glycoside or glycosides in the tobacco tops. This fraction contained 2.86 millimols of a trichloro-compound and yielded on acetylation 1.12 grams of crude product obtained from absolute alcohol in four fractions, three of which melted (without recrystallization) at 134° to 138° C. (uncorr.) while

a fourth fraction (0.38 g.) melted at 181° to 182° C. The latter fraction after two recrystallizations from absolute alcohol gave 0.20 g. melting at 184.5° to 185.0° C. (corr.) and showed no depression in a mixed melting point determination with synthetic β-2-trichloroethylgentiobioside heptaacetate (6).

No attempt was made to acetylate all the material obtainable from the tops in this series since the purpose of the isolation was to identify the glycosides formed rather than to estimate quantitatively the relative amounts present. Enough information is available, however, to show that the glucoside was present in far larger quantities than the gentiobioside and in this respect the tops in this series differed from the leaves studied in a later experiment in which relatively larger quantities of gentiobioside were isolated.

Isolation of β-2-trichloroethylgentiobioside acetate from the roots. A purified preparation from the expressed juice from the roots which contained 14.46 millimols of a trichloro-compound, based on analysis for non-ionic chlorine, was acetylated with 150 cc. of pyridine and 80 cc. of acetic anhydride. Yield of crude acetate melting at 176° to 181° C. (uncorr.), was 9.40 g. This product, like that obtained from the tops, was rather difficult to purify, but repeated recrystallizations from absolute alcohol gave a product with a constant melting point of 184.5° to 185.0° C. (corr.). This gave no depression in a mixed melting point determination with synthetic β-2-trichloroethylgentiobioside heptaacetate (6). Specific rotation was $[\alpha]_D^{26°} = -28.3°$ (concn., 3.685 g. in 100 cc. $CHCl_3$). The specific rotation of the synthetic compound has been found to be $-28.7°$.

Analysis. Calcd. for β-2-trichloroethylgentiobioside heptaacetate, $C_{28}H_{37}O_{18}Cl_3$: Cl, 13.85. Found: Cl, 13.77.

No evidence for the presence of β-2-trichloroethyl-D-glucoside in the roots was obtained although the various fractions were examined carefully with this end in view. Experiments with a number of other solanaceous species [see abstract (9)] have shown that mixtures of the acetates of the β-glucoside and β-gentiobioside of trichloroethyl alcohol are readily separated by fractional crystallization from absolute ethanol. It therefore seems that tobacco roots grown in the presence of chloral hydrate accumulate only β-2-trichloroethylgentiobioside.

Second Series

With a second series both the gentiobioside and glucoside were obtained directly in crystalline form from the leaves of treated plants. The plants used for these isolations were those which made up the experiment on the effect of chloral hydrate on growth, described in a previous section. After the completion of the growth test the cultures were thoroughly washed through with tap water and continued without further treatment, except

for the addition of nutrients, for 18 days. The addition of 0.7 millimol of chloral hydrate to each culture six times weekly was then started except for the plants which had previously been grown in the highest concentration of chloral hydrate in the drip culture series. The latter plants were also included in these treatments after a lapse of about two weeks. When this experiment was started it was thought that continued addition of 0.7 millimol (0.116 g.) six times per week would eventually kill the plants and determination of the amount of glycoside present when the plants died was planned. However, growth continued under these treatments and although the lower leaves died and upper leaves showed browning along the edges, the plants developed to maturity and produced viable seed (98 per cent germination). When the plants were sampled 37.8 millimols (6.25 g.) of chloral hydrate had been added to each culture in the case of three-fourths of the plants in the series and the others had received 30.1 millimols. At sampling time the leaves, stems, and fruits were spread out in thin layers and allowed to dry at room temperature. The roots were not sampled.

After the material had become thoroughly dry it was ground to a powder and analyses made for non-ionic chlorine. The amount of non-ionic chlorine found in the various tissues expressed as millimols of a trichloro-compound per 100 grams of air-dry weight was as follows: leaves, 31.9; stems, 3.7; fruit coats, 7.9; and seeds, 0.0. It is seen that by far the largest amount was present in the leaves, the stems containing only about one-eighth as much. Exclusion of the added chemical from the seed is of interest in view of the presence of such large amounts in the leaves.

The indicated content of 31.9 millimols of a trichloro-compound per 100 grams of dry weight of leaves corresponds to 9.9 g. if calculated as β-2-trichloroethyl-D-glucoside and 15.1 g. if calculated as the gentiobioside. Actually, a mixture of the two glycosides was present and on the basis of the isolations reported below, about 69 per cent on a weight basis consisted of gentiobioside and 31 per cent of glucoside. Taking into account the differences in the molecular weight of these two glycosides and assuming that the ratio of the two substances actually isolated corresponds to the ratio present, these leaves contained about 12.9 per cent of trichloroethyl glycosides. This amount is so large that it is evident that these non-naturally occurring compounds comprised major constituents, quantitatively, of the tobacco leaves produced. By the isolation procedure employed, a total of crystalline glycosides and glycoside acetates corresponding to 9.8 per cent of the air-dry weight was obtained from 386 grams of leaves; this represents a yield of 76 per cent of the calculated amount. In view of the expected losses associated with the many operations involved in these isolations, this result indicates that in all probability all or almost all, at least, of the absorbed chloral hydrate was present in the leaves in the form of the β-glucoside or β-gentiobioside of trichloroethyl alcohol.

Isolation of crystalline β-2-trichloroethyl-D-glucoside. After some preliminary experimentation, since isolations had not previously been attempted on dried material, the following isolation procedure was followed. The tobacco leaves (386 g.) were covered with 80 per cent ethyl alcohol and the mixture heated to boiling. The material was then filtered and washed and the powdered leaves again extracted with boiling 80 per cent alcohol. This was repeated five times. The combined extracts were concentrated *in vacuo* to remove the alcohol and precipitated with an excess of lead acetate, filtered, and the filtrate precipitated with mercuric acetate. After filtration the excess metals were removed by H_2S, the metal sulphides filtered off and the filtrate concentrated to about 450 cc. *in vacuo*. The solution was then extracted in a continuous extractor with ethyl ether. After running the extractor for a few hours the ether extract was concentrated to remove the ether, the residue taken up in water and extracted a few times in a separatory funnel with $CHCl_3$. The $CHCl_3$ removed oily material which interfered with the crystallization of the β-2-trichloroethyl-D-glucoside from an alcoholic solution of the ether extract, a procedure which had been followed successfully with preparations from the dandelion (8). After extraction with $CHCl_3$ the aqueous solution remaining was concentrated under vacuum to remove the water, the residue taken up in a little absolute ethanol, and precipitated by the addition of petroleum ether. The crystals obtained from the first two hours of ether extraction amounted to 0.69 g. and melted at 150° to 152° (uncorr.). Extraction with ether was continued for further periods of from three to five hours at the start and later the flask was changed only every 24 hours. After the first three extraction periods purification by shaking with $CHCl_3$ was no longer necessary to obtain the crystalline glucoside. Extraction with ether was continued until no further crystalline product was obtained; this required about 140 hours. The total amount of crystalline product isolated, melting within a few degrees of the melting point of synthetic β-2-trichloroethyl-D-glucoside (8), was 8.61 g. By acetylation of mother liquors from which further crystalline glucoside could not readily be obtained, crystalline acetate corresponding to an additional 2.81 g. of the glucoside resulted.

For analysis, 3.06 g. of the crude glucoside was recrystallized twice from ethyl acetate to give 1.50 g. melting at 152.5° to 153.5° (corr.). No depression was observed in a mixed melting point determination with synthetic β-2-trichloroethyl-D-glucoside (8). Specific rotation was $[\alpha]_D^{25°} = -39.9°$ (concn., 2.345 g., H_2O), as compared to $-39.7°$ reported for the synthetic compound.

Analysis. Calcd. for β-2-trichloroethyl-D-glucoside, $C_8H_{13}O_6Cl_3$: Cl, 34.14. Found: Cl, 34.12.

Isolation of crystalline β-2-trichloroethylgentiobioside. After the tobacco leaf concentrate failed to yield further glucoside when extracted with ethyl

ether, extraction in the continuous extractor was continued with ethyl acetate as the solvent. The ethyl acetate extracts on standing for a few days showed deposits of crystals on the bottom of the flasks. These were collected by pouring off the ethyl acetate, dissolving the crystals in a small amount of absolute alcohol, concentrating under reduced pressure to a small volume, and allowing to crystallize for some hours at room temperature. The crystals thus obtained, when heated for a melting point determination, decomposed at 190° to 195° (uncorr.). Concentration of the ethyl acetate solutions from which the crystals had originally separated failed to yield further crops of crystals, apparently because of the impurities present, since acetylation yielded considerable quantities of the acetate of β-2-trichloroethylgentiobioside. Extraction of the gentiobioside by ethyl acetate was very slow and it took a number of days to obtain as much as one gram of the crude gentiobioside. Nevertheless, the ethyl acetate extractions were continued until no further material was obtained; this took about two months. At the end of this time there had been obtained a total of 13.53 g. of the crystalline gentiobioside and an amount of acetyl derivative equivalent to an additional 12.66 grams.

β-2-Trichloroethylgentiobioside has not previously been obtained in crystalline form and it was thus necessary to establish definitely the identity of the crystals obtained. Analyses for carbon, hydrogen, and chlorine gave satisfactory agreement with the theoretical values. Preparation of the acetyl and propionyl derivatives gave compounds identical with synthetic β-2-trichloroethylgentiobioside heptaacetate and heptapropionate (6) respectively. Furthermore, deacetylation of β-2-trichloroethylgentiobioside heptaacetate previously obtained from tomato plants (*Lycopersicon esculentum* Mill.) which had been treated with chloral hydrate gave the crystalline gentiobioside identical with that obtained from the tobacco leaves.

For analysis 1.15 g. of the crystals obtained from the ethyl acetate extraction were purified by three recrystallizations from absolute alcohol to give 0.46 g., melting with decomposition at 204° to 206° (corr.). Specific rotation was $[\alpha]_D^{25°} = -41.2°$ (concn., 4.420 g., H_2O).

Analysis. Calcd. for β-2-trichloroethylgentiobioside, $C_{14}H_{23}O_{11}Cl_3$: C, 35.50; H, 4.89; Cl, 22.46. Found:[1] C, 35.55; H, 5.02; Cl, 22.33.

Preparation of the acetyl and propionyl derivatives of the isolated β-2-trichloroethylgentiobioside. One hundred mg. of the isolated glycoside were acetylated with 3 cc. of pyridine and 2 cc. of acetic anhydride. Yield, 150 mg. After recrystallization from absolute alcohol the product had a melting point and mixed melting point with an authentic specimen of β-2-trichloroethylgentiobioside heptaacetate of 184.5° to 185.0° (corr.).

[1] Analyses for carbon and hydrogen by Dr. Edward K. Harvill.

The propionyl derivative was obtained from 250 mg. of the isolated glycoside and 5 cc. of pyridine and 4 cc. of propionic anhydride. Yield, 420 mg. Recrystallization from absolute alcohol gave the pure substance with the melting point and mixed melting point identical with that found for the synthetic heptapropionate (6).

Deacetylation of β-2-Trichloroethylgentiobioside Acetate from Tomato Tops to Yield the Crystalline Gentiobioside

Deacetylation by the method of Isbell (4) of 2.48 g. of β-2-trichloroethylgentiobioside heptaacetate previously obtained from tomato plants (6) gave 1.49 g. or 97 per cent of the free gentiobioside. After several recrystallizations from absolute ethanol the product melted with decomposition at 204° to 206° and had a specific rotation of $[\alpha]_D^{25°} = -41.1°$ (concn., 2.205 g., H_2O).

Analysis. Calcd. for $C_{14}H_{23}O_{11}Cl_3$: Cl, 22.46. Found: Cl. 22.01.

Isolation of β-2-Trichloroethyl-D-Clucoside as the Acetate from Tobacco Leaves Which Had Absorbed Chloral from the Surrounding Atmosphere

In a previous section, data were presented which showed that control plants which had been grown near plants being treated with chloral hydrate contained a small amount of non-ionic chlorine in the leaves. This indicated that the control leaves had absorbed some of the chemical which evaporated from the cultures to which chloral hydrate had been added. In order to verify this a large number of these leaves (1519 g.) were sampled and the expressed juice obtained. The leaves taken were the 7th to the 18th leaf, counting from the base of the plant, since the lower leaves were found in the analytical study to contain the largest amount of non-ionic chlorine. The lower six leaves were not included either in this sample or in the study of the distribution of the non-ionic chlorine with reference to leaf position since these leaves were in a partly dried condition. The sample taken yielded 1020 cc. of expressed juice which was found to contain non-ionic chlorine equivalent to 0.33 millimol of a trichloro-compound per 100 cc. For the isolation of the β-glycosides, the procedure which had previously been used with dandelion leaves (8) was used. The ether extract failed to give a crystalline product, presumably because of the relatively larger amounts of extraneous substances present compared to the low content of glucoside. Acetylation of the material extracted, however, yielded 0.66 grams of crude acetate melting at 141° to 142° (uncorr.). Two recrystallizations from absolute alcohol gave the pure substance melting at 144.5° to 145.5° (corr.) and showing no depression in a mixed melting point determination with synthetic β-2-trichloroethyl-D-glucoside tetraacetate.

Further extraction with ethyl acetate of the concentrated lead precipitated juice gave no crystalline product even after acetylation. Failure

to obtain any of the β-gentiobioside was somewhat unexpected since with the plants which had absorbed chloral hydrate from the nutrient medium, the β-gentiobioside as well as the β-glucoside were isolated from the leaves. Further tests with leaves which obtained their supply of chemical from the vapor in the surrounding air are necessary in order to reach any conclusions, but these results indicate that the leaves may form only the β-glucoside and that the β-gentiobioside isolated from the leaves in the other experiments may have been translocated from the roots which were found to contain only the β-gentiobioside. This would also explain why, in the two series in which isolations were made from plants treated in the usual way, relatively larger amounts of the β-gentiobioside were found in the second series, since the plants of this series were grown for a much longer period with added chloral hydrate and thus more opportunity was afforded for translocation of the β-gentiobioside from the roots. On the other hand, experiments in which plants were grown from potato tubers containing β-2-chloroethyl-D-glucoside and from gladiolus corms containing β-*o*-chlorophenyl gentiobioside showed that in these cases the glycosides were not translocated to the new growth (5). It is thus evident that further experiments are necessary in order to demonstrate whether or not the β-2-trichloroethylgentiobioside found in tobacco leaves of plants grown with chloral hydrate added to the nutrient medium is formed in the leaves or translocated from the roots.

DISCUSSION

These results add *Nicotiana tabacum* L. to the list of species which have been shown to form β-gentiobiosides when treated with chemicals which induce glycoside formation. Since gentiobiose is itself a β-glucoside [6-(β-D-glucosido)-D-glucose] and can be prepared *in vitro* from glucose (2, 3) by the use of emulsin under conditions similar to those which have resulted in enzymic syntheses of other β-glucosides, it might be expected to be rather widely distributed. On the contrary, however, aside from its quite general distribution in the rosaceous family as the sugar component of amygdalin, the reported occurrence of gentiobiose is limited to a few isolated cases. The frequency with which gentiobiose has been found to be the sugar component of the induced glycosides formed in the present series of experiments has thus been unexpected. However, with one exception (*Gladiolus* species), induced formation of gentiobioside has been limited to solanaceous species. Including unpublished results, experiments in this laboratory have involved 15 species belonging to nine different families. With seven solanaceous species tested, β-gentiobiosides have resulted in at least one of the plant organs included in the study (9). On the basis of the evidence thus far available it appears that gentiobioside formation may be a characteristic of the solanaceous family, even though there is no reported natural occurrence of gentiobiose in this family.

Glycoside formation in plants is sometimes considered as a detoxication reaction (1). The results with the added chemicals indicate that glycoside formation can take place quite generally among the higher plants regardless of whether they contain naturally occurring glycosides. If glycoside formation really results in a detoxication, as seems not unlikely, a possible practical application suggests itself in connection with attempts to use internal therapy with certain plant diseases. If the chemicals tried contain hydroxyl or potential hydroxyl groups, they might serve temporarily as toxic agents to invading organisms but they might be prevented from being too harmful to the host plant through the ability of the host to ultimately convert the absorbed chemicals to less toxic glycosides.

The results on induced glycoside formation show that it is possible to add various organic chemicals to the growing medium of plants and have relatively large quantities absorbed by the growing plants and enter into metabolic processes going on within the plant. There is no reason to limit experiments involving the addition of various organic chemicals to the nutrient medium of plants and subsequent isolation of the products formed within the plant to chemicals which are converted into glycosides. The processes by which various plants elaborate exceedingly complex organic compounds are for the most part unknown. In most cases duplication of such compounds in the laboratory must employ conditions obviously impossible in living cells. Many reactions have been postulated which it was hoped might indicate some of the steps taking place in the building up of compounds in the plant. Isolation of such postulated intermediates offers many difficulties because it would be expected that in most instances the presence of these compounds would be only transitory and the actual concentration at any one time would be very low. Some of the theoretical reactions, it would seem, could be tested experimentally by furnishing plants with certain possible intermediates and determining the fate of the introduced chemicals. Results obtained, if positive, would prove that the plants could bring about the reactions in question.

SUMMARY

Through the isolation and characterization of the crystalline glycosides and the acetyl derivatives, tobacco (*Nicotiana tabacum* L.) leaves have been shown to form β-2-trichloroethyl-D-glucoside and β-2-trichloroethylgentiobioside from absorbed chloral hydrate. The roots accumulated only the β-gentiobioside.

Crystalline β-2-trichloroethylgentiobioside, melting with decomposition at 204° to 206° C. and with a specific rotation of $[\alpha]_D^{25°} = -41.2°$ (H_2O), was obtained for the first time. Its identity was established through analysis and the preparation of the acetyl and propionyl derivatives which were found to be identical with the corresponding synthetic compounds.

Growing tobacco plants could withstand the addition of large amounts of chloral hydrate to the nutrient medium with the resultant accumulation of the β-glycosides of trichloroethyl alcohol in the leaves and roots and to a lesser degree in the stems to such an extent as to feature these glycosides as major constituents, quantitatively, of the tissues produced. In one experiment tobacco leaves were obtained which were shown by analysis to contain about 13 per cent on a dry weight basis of a mixture of β-2-trichloroethyl-D-glucoside and β-2-trichloroethylgentiobioside. Isolations carried out with a 386 g. portion yielded 76 per cent of the indicated content as the crystalline glycosides or glycoside acetates.

It is suggested that experiments involving the absorption of various organic chemicals (not limited to those converted to glycosides) by growing plants and the determination of their fate within the plant offer a technique of promise for studying the organic reactions carried out by growing plants.

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CARBON DIOXIDE STORAGE. XIV. THE INFLUENCE OF CARBON DIOXIDE, OXYGEN, AND ETHYLENE ON THE VITAMIN C CONTENT OF RIPENING BANANAS[1]

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The vitamin C content of the banana (*Musa sapientum* L. var. Gros Michel) has been reported many times with a different value for almost every investigator. This variation, from 3.0 to 20.0 mg. per 100 g. of tissue, as given in the literature reported by Harris and Poland (10, 11), has been due no doubt to the methods of analysis. The majority of these reports were made before it was known that the banana contained an enzyme system that would rapidly destroy the vitamin C content when the living system was broken up for either animal feeding or chemical tests. Ever since the presence of such a system was recognized, the proper precautions have been taken to inactivate the enzymes so that a consistent amount of vitamin C could be obtained for any stage of ripeness under normal conditions. Only in rare cases have the investigators reported vitamin C values below 10 mg. per 100 g. of tissue for the ripe, edible fruit. However, this has not been true for unripe fruit and it is believed that low values have been due to the dependence entirely upon a chemical method which has employed too weakly ionized acid for the extraction. Furthermore, the difficulty of mashing the green pulp has, in many cases, made it necessary to resort to the use of a grinder for preliminary division of the tissue, of course in absence of the acid, which allowed for the destruction of the vitamin C. On the basis of the findings in this investigation, one is justified in stating that the firm, ripe banana with yellow peel contains approximately 12 mg. and the full ripe banana with 50 per cent brown skin contains approximately 10 mg. of vitamin C per 100 g. of fresh tissue. There are many factors responsible for variations from these values and it is the purpose of this paper to present and discuss the causes for such deviations in experimental results.

MATERIAL AND METHODS

The bananas used in the main part of this investigation were obtained from the United Fruit steamer in New York City and brought directly to the laboratory. A few banana fingers were purchased at various retail stores in order to check on the vitamin C content of the fruit as handled for market purposes. The hands of fruit having 16 to 20 fingers were divided into groups of inner and outer fingers, samples were taken for determination of ascorbic acid content, and the remaining fingers, usually three to

[1] The writer is indebted to the United Fruit Company for furnishing the bananas used in this work and to Mr. G. L. Poland of the United Fruit Company Research Laboratory for suggestions during the course of this investigation.

five, were placed in 20-liter tin cans for treatment with various gas mixtures. The gas mixtures containing oxygen, carbon dioxide, and nitrogen from cylinders of the compressed gases were made up and transferred into the cans containing the bananas as has been previously described (16). Control fruit received the gas mixture of 20 per cent of oxygen and 80 per cent of nitrogen made up in the usual way. In addition there was placed in some of the control containers a beaker containing 300 cc. of 4 per cent sodium hydroxide with pieces of cheesecloth suspended in it to increase the liquid surface area to absorb the carbon dioxide produced through the respiration of the bananas. Alkali in this percentage concentration causes no practical interference with the relative humidity in the container. The control container, though sealed like the other containers, had a capillary tube opening in the top to prevent a vacuum from forming in the container. Later it was found unnecessary to seal the cover on the control container in order to keep a high relative humidity about the fruit.

The original gas mixtures as well as the alkali solutions were replaced every 24 hours. Gas analyses for both oxygen and carbon dioxide concentration in all the treatments were made at frequent intervals. The oxygen concentration was never below 16 per cent by volume except when so desired in some experiments. In those cases where the control fruit was held with alkali the carbon dioxide concentration was not measurable with an Orsat apparatus.

The temperature of the bananas was held at 19° C. (66.2° F.) during the period of the investigation in the years 1937 and 1938 by placing the containers in a chamber, the temperature of which was thermostatically controlled by a slow current of air circulated over the heating elements and throughout the chamber.

During the investigations in 1941 and 1942, the bananas were placed directly in the ripening chamber and the temperature was varied from 21.5° C. to 17° C. The higher temperature was used at the start of the test when the fruit was green and hard. As ripening progressed the temperature was decreased as needed in order to maintain as nearly as possible the normal conditions for commercial ripening. At the same time the relative humidity of the chamber was held at approximately 90 per cent during the early stage of ripening and was reduced to approximately 75 per cent with later stages of ripening.

All fruit was weighed at the beginning and again at the end of each treatment in order to determine the effect of weight loss on the vitamin C content. In all treatments where the fruit was held in containers, the weight loss varied from 1 to 3 g. with 200 to 400 g. of fruit per 24-hour period with no correlation between treatment and weight change. The fruit held in the room usually lost between 3 and 6 g. for the 24-hour period when the humidity was maintained at the optimum for ripening. Moisture deter-

minations were made on the edible pulp tissue at the various stages of ripening in order to calculate the vitamin C on both the fresh and dry weight basis, but this was discontinued when it was found that the same general trend in the results was obtained on either basis.

Determination of ascorbic acid. The method for sampling the banana is to take an unpeeled finger, wipe or wash off any foreign material, and quickly make two cuts crosswise of the finger, through peel and pulp, about one inch or so apart, depending upon the size of the fruit. This section is rapidly peeled and weighed and placed in the acid medium so that all of the surfaces are covered by the acid during grinding. The duplicate sample is next taken by first cutting and discarding about one-half inch of the banana having the cut surface that has been standing, and taking the next inch or so. There is some loss of ascorbic acid at the cut surfaces of the fruit, but at the most this amounts to 0.3 to 0.4 mg. per 100 g. of tissue if both of the samples are not handled with the utmost expediency. However, duplicate, triplicate (19), or even quadruplicate samples that check consistently may be obtained if the fruit is quickly handled as outlined above.

Tests were made on uniformity in the ascorbic acid content of fingers from various parts of a hand of bananas. Fruit of any one lot obtained in 1937 to 1941 was found to be very uniform in vitamin C content either in respect to different hands, if all are the same in color, or in respect to inner and outer fingers, or in respect to different parts of the finger (19). Determinations made on the fingers of one partially ripe hand gave values that varied between 12.7 and 13.3 mg. ascorbic acid per 100 g. when comparing right, center, and left portion of the hand, or between hands cut from the same stalk of bananas. The variation between inner and outer fingers was determined to be between 0.0 and 0.3 mg. ascorbic acid. Yellow brown-flecked fruit was found uniform in vitamin C content irrespective of the position of the fingers on the hand.

The method used for the determination of vitamin C was the same as that previously reported (19). The extracting medium used in these experiments consisted of equal parts of 1 N or 2 N sulphuric acid and N/4 metaphosphoric acid. For the most part the acid extract of the banana was titrated with 2,6-dichlorophenolindophenol until the end point, as evidenced by a pink coloration of the extract, was visible. The dye solution, made up every third day, was standardized daily by titration of pure ascorbic acid and by the method of Ballentine (1). Comparisons were made between the visual titration and a potentiometric determination of ascorbic acid in the banana extract with the result that the values obtained by both methods were in good agreement. The apparatus used was the mercury-platinum electrode devised by Harris and co-workers (8) in conjunction with a continuous reading device having two triodes in a

wheatstone bridge arrangement, which is merely an elaboration of the Goode (6) electro-titration apparatus.

Investigations led to the conclusion that the best results in the determination of vitamin C of banana tissue are to be obtained with one normal or stronger sulphuric acid. This acid rapidly inhibits enzymatic action and eliminates the necessity for the treatment of the extract with hydrogen sulphide in order to reduce the ascorbic acid that may have been oxidized during the process of extraction. Results with lower concentrations of sulphuric acid and with various concentrations of other acid media also bear out this conclusion. Weaker sulphuric acid concentrations such as N/2 and N/4 mixed with metaphosphoric acid gave lower values for the vitamin C content of the banana than were obtained with 1 N H_2SO_4. These values were 11.0 and 14.9 per cent lower for N/2 and N/4 H_2SO_4 respectively. When these weaker acid extracts were treated with hydrogen sulphide in the usual manner to reduce any partially oxidized ascorbic acid, there was some increase in the amount of ascorbic acid present so that the loss was now only 4.1 and 3.3 per cent respectively. At the same time there was no change in the ascorbic acid content of the 1 N H_2SO_4 extract upon treatment with hydrogen sulphide. In another test the N/2 and N/4 H_2SO_4 extracts were brought to a pH of 5.3 by the addition of sodium hydroxide and there was found a progressively lower titration value with increase in pH. However, upon treatment of these extracts with H_2S, the titration values for both solutions increased, the solution with the stronger acid giving the higher titration which indicated that weakly acid solutions should not be used for the extraction of vitamin C from the banana.

Tests were made on green fruit using acetic acid with N/4 HPO_3 as the extracting medium in order to understand the discrepancy between the vitamin C values reported (11, 12) for this medium and the higher values obtained by the use of sulphuric acid. In these cases the percentage concentrations of acid were varied in steps of 10 up to glacial acetic acid. In every case there was a progressive increase in the amount of ascorbic acid in the extracted solution up to 40 per cent acetic acid. Even in this case reduction with H_2S brought about an additional 15 per cent increase in the amount of ascorbic acid present in the extract which then was only 8 per cent below that obtained with the 1 N H_2SO_4 extract. The 10 per cent acetic acid extract gave a value that was 24.3 per cent below that of the sulphuric acid. Unfortunately, concentrations of acetic acid above 20 per cent are very hard to titrate with the 2,6-dichlorophenolindophenol while concentrations above 40 per cent are impossible to titrate. The acetic acid retards discoloration of the indicator with progressive concentration. Electrometric titrations of glacial acetic acid and 1 N H_2SO_4 extracts of banana tissue gave the same values for the ascorbic acid content of duplicate samples of yellow ripe fingers.

Other methods used in attempts to determine the ascorbic acid content of the banana have given very poor results. Whenever a method requires the extraction of the ascorbic acid from the banana in a solution approaching pH 5 to 7, destruction of the vitamin results so rapidly that far too low a value is obtained. Also, similar low values are obtained in those methods that necessitate much manipulation of the tissue during the process of extracting of the vitamin.

As reported (19), it has been possible to recover 100 per cent of synthetic ascorbic acid added to banana tissue at the time of mashing in 1 N sulphuric acid. This shows that the ascorbic acid is not oxidized during the mashing with the acid and indicates that the reduction of the banana extract with hydrogen sulphide is unnecessary.

EXPERIMENTAL RESULTS

A survey of the data given in Table I for years 1937, 1938, and 1941, showed that the green bananas, color numbers 1 to 3, contained approximately 15 mg. of vitamin C per 100 g. of pulp tissue and that there occurred a gradual decrease in vitamin content as the fruit ripened. Bananas with yellow peel, color 6, contained about 12 mg. and those with 50 per cent brown peel, color 8, had approximately 10 mg. of vitamin C. As ripening progressed from color 8 to 11, the vitamin content decreased to about 8 mg. when the fruit had a dark brown or black peel and the edible portion had a disagreeable flavor. This decrease in vitamin C content of the banana from the green to full ripe stage represented a loss of about 47 per cent. However, at the desirable eating stage, represented by color numbers 6 to 8, the reduction in vitamin C content was only 33 per cent lower than that contained in the hard green fruit.

The data in columns 3 to 14 inclusive, Table I, have been averaged for each stage of ripeness of the fruit and these averages are given in the last column. These values represent in general the approximate vitamin C content of bananas at various stages of ripening as indicated by the peel color and condition of the pulp shown in column 1. Since this fruit was held in as nearly normal commercial ripening conditions as possible in respect to temperature, humidity, and ventilation after it was received, it may be assumed that these values represent the actual vitamin C content of bananas available in the market. Many tests were made on bananas obtained at various stages of ripeness from the local retail markets during the years 1937 and 1938 which substantiated this idea.

The remaining portion of the data given in columns 15 to 27 in Table I, was not included in the calculation of the average values because of the delay in handling the fruit during the shipping emergency in 1942. However, if all of the data for each stage of ripeness are averaged and these compared with those average values in column 28 for 1937, 1938, and 1941

TABLE I

VITAMIN C CONTENT OF BANANAS AT DIFFERENT STAGES OF RIPENING

Peel color and condition of pulp	Color number	Milligrams of ascorbic acid per 100 grams of edible banana tissue — Year and month analyses were made: 1937			1938						1941 Dec. Composite sample*			1942	Mar.** Hand number and position of finger on hand†												Av. of columns 3 to 14
		Sept.	Oct.	Nov.	Jan.	Jan.	Jan.	Jan.	Jan.	Feb.	A	B	C	Mar.	1 I	1 O	2 I	2 O	3 I	3 O	4 I	4 O	5 I	5 O	6 I	6 O	
Dark green	1	14.7	15.5	14.1	14.4	14.4	13.3		13.7	14.1																	14.3
Light green	2	15.6	16.0	14.2	15.8	14.8	14.4							15.6	11.0	12.0	12.0	12.1	13.5	13.4	12.6	13.5	12.4	12.5	11.3	11.4	15.1
Yellowish-green	3		14.9		16.5	15.4	15.0	14.8		13.1					13.3	12.9	15.0	14.6	16.1	15.4	16.1	16.1	13.5	13.5	14.6	15.4	14.9
Greenish-yellow, more yellow than green	4	13.9		13.6	15.4				13.5	12.8				15.0	15.5	14.8	16.6	15.9	15.3	14.4	16.8	16.3	16.1	15.5	15.4	15.0	13.8
Yellow, green tip	5	13.5	12.5		12.7	13.9	12.7		13.5		12.5	12.1	11.8	14.5	14.1	14.3	14.5	13.9	15.9	16.1	14.7	14.2	13.8	14.9	13.9	14.4	12.6
Full yellow	6	13.1		11.8		12.7	10.9	11.5	13.6		12.4	12.4	11.6	13.2	11.3	11.8	12.6	13.0	11.8	11.4	13.0	14.0	12.0	12.2	12.1	11.9	12.2
Yellow brown-fleck	7		10.1	11.4				11.3			10.6	10.8	10.6	12.2	10.6	10.6	10.7	10.8	10.1	11.0	10.2	10.2	10.9	10.8	10.1	10.2	10.8
50% Brown, pulp soft	8		10.1	10.9			9.8				10.1	10.1	10.2	9.9													10.2
Brown, pulp very soft	9		9.9	10.3							8.2	8.5	7.8	8.8	9.4	9.1	—	—	9.0	8.7	8.2	9.2	8.7	8.0	8.6	8.7	8.9
Dark brown, light brown tip, slight fermented flavor	10										8.0	8.1	7.4														7.8
Black, soft, strong fermented flavor	11										7.7	7.7	7.7	7.6					9.0			9.0					7.7

* Each composite sample consisted of one finger from each of four hands. Each hand was taken from the center of a stalk of bananas.
** Due to an emergency, this fruit was held aboard ship for three days after reaching port.
† I represents inner whorl, O—outer whorl of fingers of hand. Each hand taken from a separate stalk of bananas.

fruit, there will be found only slight divergence in the vitamin C content at the early or green peel stages of ripening. From colors 5 to 11 there was very little difference in the averages, whether 1937, 1938, and 1941 data were used, or all of the data in the table were included. However, it may be observed that in most cases the vitamin C content of color 1 fruit was lower than that for color 2 fruit. The real reason for this result was not completely understood but the 1942 investigation did offer some explanation for these observations. It was found during the emergency when bananas were held in storage for extended periods that the vitamin C content of the green fruit was not as high as that found in previous years. The fruit analyzed in March 1942, columns 16 to 27, Table I, had been held aboard ship in port for three days during which time it had colored slightly yet it contained a somewhat lower amount of vitamin C than usually found in green fruit. When this fruit was held under good ripening conditions it developed a high content of vitamin C—in fact, higher than usual for color 4 fruit—which then decreased upon further ripening just as usually found under normal conditions of handling. This result indicated that those slightly lower values obtained with green fruit just removed from the ship in previous years may have been either a result of some condition of shipping or that the green banana just removed from the plant may have had a low rather than a high vitamin C content which reached a maximum with ripening. Although it was not possible to answer the second portion of this statement, one test early in the course of this investigation provided information relative to the first portion. Some green fruit was held in an unventilated room for about 40 hours previous to analysis for vitamin C with the result that the first values were quite low, but when held in a ventilated chamber the fruit recovered its usual vitamin C content during the period of ripening. This led to the study of the effect of oxygen, carbon dioxide, and ethylene on the vitamin C content of ripening bananas.

STORAGE IN OXYGEN

Green bananas were stored in various percentages of oxygen during a ripening period of 186 hours at 19° C. Fruit held in 0, 5, and 10 per cent of oxygen colored very slowly, being at color 3 at the end of the test, and showed by five determinations a slight increase in vitamin C content comparable with the increase usually found for this stage of ripening. The bananas held in 20 per cent of oxygen were at the full yellow, color 6, stage by the end of 186 hours and contained 10.9 mg. vitamin C (see column 8, Table I). Ripening progressed more rapidly with increased percentages of oxygen so that the fruit in 100 per cent of oxygen reached color 6 by 114 hours and contained 13.8 mg. of vitamin C. Although bananas in 40 and 60 per cent oxygen did not ripen as fast as those in 100 per cent O_2, they ripened more quickly than those in 20 per cent O_2 and

maintained a higher vitamin C content. When at the end of 186 hours the bananas exposed to high oxygen were at color 8 and those exposed to 20 per cent O_2 were at color 6, the fruit contained the following amounts of vitamin C: 13.6 mg. for 100 per cent O_2, 12.0 mg. for 40 and 60 per cent O_2, and 10.9 mg. for 20 per cent O_2.

At the end of the 186-hour period all remaining fruit was removed from the treatment and held in a ripening room exposed to normal atmosphere for an additional period of 144 hours. When analyzed at the end of this period, fruit previously held in 5, 10, 80, and 100 per cent of oxygen contained from 11.4 to 11.9 mg., fruit in 40 per cent O_2 contained 10.9 mg., fruit in 20 per cent O_2 contained 9.8 mg., and fruit in 0 per cent O_2 contained 12.7 mg. of vitamin C. At the time of these analyses the fruit held in low oxygen had ripened to color 5, that in high oxygen to color 9, and the fruit held in 20 per cent oxygen was color 8. Although the fruit held in the absence of oxygen was slow to ripen while it maintained a high vitamin C content, it was of poor flavor, being quite acid to the taste. It was apparent from these results that the lack of oxygen could not have been the limiting factor in the production of vitamin C in the green bananas during early storage.

STORAGE IN CARBON DIOXIDE

Continuous treatment. Since varying the concentration of oxygen did not greatly change the amount of vitamin C in the ripening fruit the next tests were made with increased concentrations of carbon dioxide in the storage atmosphere because this gas would be expected to accumulate about the ripening fruit during storage. When various amounts of carbon dioxide were added to the storage atmosphere there was found, as shown in Figure 1, a decided loss in vitamin C content of the fruit. Not only did the loss become greater both with increase in concentration of carbon dioxide and length of treatment up to the sixth day, but the carbon dioxide produced by the respiration of the bananas also brought about a reduction of the vitamin C content of the fruit. Apparently the respired carbon dioxide is most effective in reducing the vitamin C content during the early or green peel stages of ripening, becoming less effective with advancing stages of ripening. Bananas in the closed container produced the following maximum percentages of carbon dioxide for each 24 hours of storage: 5 per cent for each of the first two periods, 4 per cent for each of the third and fourth periods, and 3 per cent for each of the remaining five periods. Since the gases were changed every day, there remained sufficient oxygen, 16 per cent or greater, to maintain respiration of the fruit.

Although the fluctuating concentration of respired carbon dioxide caused only 3 to 20 per cent reduction in the vitamin C content during six days' storage of the green fruit as compared with similar fruit held in normal atmosphere, this CO_2 effect could reach major proportions if more

FIGURE 1. The effect of various concentrations of carbon dioxide on the ascorbic acid content of the banana during storage at 19° C. (66.2° F.).

of the gas was allowed to accumulate. Continuous treatment with as little as 8 per cent CO_2 during this period caused from 30 to 50 per cent reduction in the vitamin C content while 24 to 60 per cent CO_2 caused a reduction of 80 per cent of the vitamin C content. Thus it is apparent from the results shown in Figure 1 that carbon dioxide is effective in reducing the vitamin C content of ripening bananas.

Prolonging the CO_2 treatment from the sixth day produced some very interesting results which were substantiated by repeated tests. After a period of continued reduction in the vitamin C content resulting from the CO_2 treatment, the bananas began to show by analyses an increase in vitamin C content in spite of the presence of carbon dioxide (see Fig. 1 for the 6- and 9-day periods of treatment). The upward trend of the curves for the CO_2-treated fruit indicate that if the experiment was continued with sufficient fruit to make analyses beyond the ninth day the CO_2 curves would approach the curve for the control. This was done in other tests with two concentrations, 25 and 50 per cent, of CO_2 besides the control treatment with the result that on the third day of treatment the analyses showed 14.0, 6.6, and 4.5 mg. of vitamin C for the control, 25, and 50 per cent CO_2 treatments, respectively. On the eleventh day of treatment when the control fruit was at color 9 and contained 10.3 mg. of vitamin C, the fruit in 25 per cent CO_2 was color 8 and contained 9.6 mg. of vitamin C while the fruit in 50 per cent CO_2 was injured (brown skin specked with yellow and slightly soft), and contained 8.7 mg. of vitamin C. Continued treatment resulted in the usual softening and reduction of vitamin C in the control fruit as indicated in Table I together with a paralleled softening and reduction in the vitamin C content of the CO_2-treated fruit.

Ripening of the bananas was retarded by treatment with carbon dioxide. Usually the retardation, especially color changes and rate of respiration, was not very noticeable until 30 per cent or more CO_2 was used. With 50 to 60 per cent CO_2 the softening and color changes were retarded for some time, then the fruit showed injury by either being pale yellow in color upon ripening or developing brown areas in the peel and the pulp becoming soft without losing all of its starchy flavor. Treatment of the bananas with CO_2 caused the green fruit to lose its astringency possibly by altering the tannin compounds. Microscopic examination of the starch grains in bananas treated with carbon dioxide showed that the starch broke down in an abnormal manner during the treatment even at the time when the color changes in the peel would indicate very little ripening taking place. Those exposed to carbon dioxide decomposed into a soluble mass without showing the etching characteristic in grains during normal breakdown.[2] On the ninth day of treatment of the fruit used in the experiment for Figure 1, the color numbers were as follows: Control, No. 8; 8 per cent CO_2, No. 6;

[2] From results of tests made by Dr. Sophia H. Eckerson.

24 per cent CO_2, No. 5; and 60 per cent CO_2, No. 3. Because of the lack of comparable fruit this test was discontinued on the ninth day but other tests have shown that if the fruit had been removed from the CO_2 to normal atmosphere all except that exposed to 60 per cent CO_2 would have ripened to normal appearance and flavor.

During the period of ripening from color No. 2 to color No. 8, the pH

TABLE II

CHANGES IN pH OF BANANAS DURING RIPENING IN AIR AND TREATMENT WITH CARBON DIOXIDE AT 19° C. (66.2° F.)

Treatment	pH of edible pulp after hours of treatment					
	48	72	96	120	144	216
Control in room*	5.4	5.4	5.4	5.0	4.7	4.7
Control in container	5.4	5.5	5.4	5.0	4.8	4.6
8% CO_2	5.4	5.4	5.5	5.3	4.9	4.7
40% CO_2	5.4	5.5	5.5	5.3	4.9	4.9
60% CO_2	5.7	5.7	5.9	5.5	5.3	5.2

* Banana peel color #2 at 48 hours and color #8 at 216 hours; the same fruit as used in Figure 1.

of the edible portion of the banana changes from about 5.4 to about 4.7 as shown in Table II. These values vary somewhat with different lots of fruit. When the fruit is exposed to high concentrations (40 to 60 per cent CO_2), the pH of the tissue becomes higher than the pH of the control fruit and remains so even to the time when the fruit becomes ripe. These re-

TABLE III

PERIOD OF TREATMENT AND COLOR OF FRUIT ALTERS THE INFLUENCE OF CARBON DIOXIDE ON THE ASCORBIC ACID CONTENT OF BANANAS HELD AT 19° C. (66.2° F.)

Treatment*	Mg. ascorbic acid per 100 g. of fresh tissue					
	Color number of fruit and hours of treatment					
	2**		4**	6**		
	22	48	48	42	72	
Control in room	16.2	13.4	13.9	12.4	12.3	12.4
Control in container	15.9	11.2	12.5	12.4	—	—
10% CO_2	—	8.1	8.9	11.5	11.5	11.5
15% CO_2	12.5	—	—	—	—	—
20% CO_2	—	6.0	9.2	11.7	—	—
30% CO_2	12.4	4.7	5.0	11.5	—	—
50% CO_2	—	—	—	—	12.0	11.8
60% CO_2	12.4	7.0	5.0	—	—	—

* Oxygen concentration was maintained between 16 and 21 per cent during all treatments.

** See columns 1 and 2, Table I, for description of color of fruit corresponding to these numbers.

sults in Table II are additional evidence that the CO_2 has a decided effect upon the metabolism of the banana.

Bananas allowed to ripen in normal atmosphere until the peel color was characteristic of color numbers 2, 4, and 6, were then exposed to carbon dioxide for various periods to determine how the gas would affect the vitamin C content. As shown in Table III, there is a relationship between concentration of CO_2, hours of treatment, and color of fruit at time of exposure to carbon dioxide. Fruit at color 2 when placed in 15 or 60 per cent CO_2 for 22 hours lost only 23 per cent of its vitamin C while the fruit exposed to CO_2 for 48 hours lost as much as 48 per cent of its vitamin C and in this test the carbon dioxide produced by the fruit caused a substantial reduction in the vitamin C content. Bananas at color 4 were likewise affected by the presence of various percentages of carbon dioxide. With riper fruit (color 6), however, little or no change in vitamin C content resulted from treatment with 50 per cent CO_2 for as long as 72 hours.

Intermittent treatment. Bananas are usually stored in ripening rooms or fruit warehouses for short periods of time; therefore, the fruit would not be exposed to carbon dioxide for long periods. However, the experimental results have shown that for such periods of two to four days' exposure, carbon dioxide may greatly reduce the vitamin C content. Therefore it was necessary to determine if there could be a recovery of this vitamin when the fruit was removed to normal atmosphere. For this purpose, experiments were set up to duplicate as nearly as possible such conditions as could possibly occur in the handling of bananas and the results of one such experiment are shown graphically in Figure 2.

Green bananas treated with 10, 25, and 50 per cent CO_2 for two days at 19° C. were reduced in vitamin C content from 14.4 mg. to 10.8, 10.2, and 8.8 mg. respectively, while a similar lot of fruit showed a further reduction in vitamin C content to 9.3, 7.3, and 4.4 mg. during four days' treatment with 10, 25, and 50 per cent CO_2. At the same time the lot of fruit held in normal atmosphere at the same temperature gave the usual slight increase and then slow reduction in vitamin C content as ripening progressed. When the fruit was removed from CO_2 to normal atmosphere there occurred a fairly rapid recovery of the vitamin C content so that when the fruit became edible by the tenth day of storage (see Fig. 2), it contained as much and in some cases slightly more vitamin C than the fruit ripened in a normal atmosphere. Also it may be observed in Figure 2 that the fruit treated with 50 per cent CO_2 recovered and maintained a higher final value for vitamin C than fruit in any of the other treatments. This is an especially interesting fact since this fruit also ripened faster and at the 10- and 11-day period of storage it was at color 7 while all other CO_2-treated (10 and 25 per cent) fruit was at color 6 and the control fruit was at color 5. It was apparent that when the fruit received only short

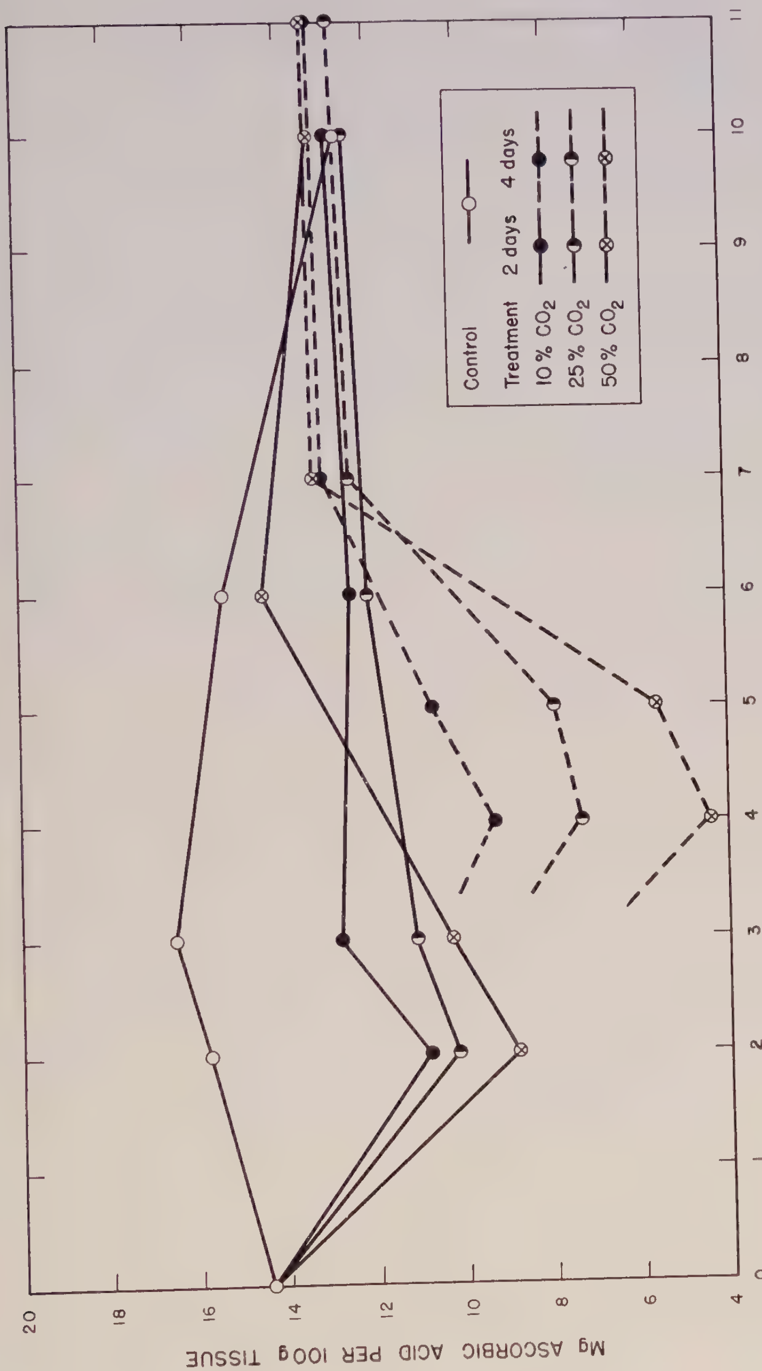

FIGURE 2. The effect of a 2- and 4-day period of storage of bananas in various concentrations of carbon dioxide on the ascorbic acid content of the fruit during ripening at 19° C. (66.2°F).

periods of treatment with carbon dioxide, and was then removed to normal air, its rate of ripening was accelerated rather than retarded as was observed when the CO_2 was present for long periods of time. And with this CO_2-stimulated rate of ripening, there may also be a stimulated rate of production of vitamin C which might explain why in a few cases in 1942 the writer found yellow ripe bananas (color No. 6) on the market to contain as much as 16 mg. of vitamin C rather than the usual values reported in Table I.

Thus it is important, if one is to preserve the vitamin C content, to store bananas in atmospheres relatively free of carbon dioxide or at most in storage atmospheres containing CO_2 for only short periods of time. However, closed storage is necessary under commercial conditions for prompt and quality ripening of green fruit in order to maintain the desired ripening temperature and to hold the ripening gas (ethylene) about the fruit. Under these conditions, carbon dioxide must accumulate, but even so, it need not be permanently detrimental to the vitamin C content of the bananas because after the fruit begins to ripen it is exposed to a moderate amount of ventilation in order to complete the ripening process. This exposure to air, relatively free of carbon dioxide, is made well in advance of the time the fruit is ripe enough to eat. Therefore, the banana could regain its usual content of vitamin C before it is used by the consumer.

STORAGE IN MIXTURES OF ETHYLENE AND CARBON DIOXIDE

The vitamin C content of green bananas stored for 67 hours in mixtures of ethylene and carbon dioxide was greatly reduced as shown by the data in Table IV. However, when the fruit was removed from treatment to normal atmosphere, there occurred as ripening progressed a gradual increase in the vitamin C content. At the time when the fruit reached an edible stage it contained a high content of vitamin C and in most cases as much as the untreated fruit at the same stage of ripeness. Thus it is evident that the combination of ethylene with carbon dioxide in the storage or ripening rooms would not be permanently detrimental to the vitamin C content of the fruit upon ripening.

STORAGE IN ETHYLENE WITHOUT CARBON DIOXIDE

Green bananas exposed to ethylene in concentrations of 1 to 1000 and 1 to 2000 parts of air for 67 hours maintained a high vitamin C content comparable with the fruit that was not treated. As shown in Table IV, the treated fruit upon removal to normal air, with ventilation to remove accumulated ethylene, ripened slightly faster than the control fruit and maintained an equivalent vitamin C content. With continued storage in normal air the ethylene-treated fruit, at a desirable eating stage, contained somewhat more vitamin C than the ripe fruit that had not been

TABLE IV

ASCORBIC ACID CONTENT OF BANANAS EXPOSED DURING STORAGE TO ETHYLENE AND MIXTURES OF ETHYLENE AND CARBON DIOXIDE AND SUBSEQUENTLY ALLOWED TO RIPEN IN NORMAL ATMOSPHERE AT 19° C. (66.2° F.) (1938 FRUIT)

Treatment*	Ascorbic acid, mg. per 100 g. of tissue and color number of fruit					
	At end of 67 hrs. of treatment		After removal to air for 24 hrs.		After removal to air for 96 hrs.	
	Mg.	Color No.	Mg.	Color No.	Mg.	Color No.
Control in air	13.5	3†	13.6	5	11.6	7
Control in sealed container**	14.3	3†	13.2	5	10.4	7
Ethylene 1:1000**	12.4	5	13.3	5†	12.2	7†
Ethylene 1:2000**	13.7	4†	13.5	5†	12.8	7†
10% CO_2	7.1	3	9.1	4	9.8	6†
10% CO_2 and ethylene 1:2000	6.9	4	9.1	5†	11.7	7
50% CO_2	3.8	2	6.0	3	12.1	5
50% CO_2 and ethylene 1:2000	5.1	2	5.6	4	10.7	5

* Oxygen concentration was maintained between 16 and 21 per cent during all treatments.

** Carbon dioxide absorbed by 4 per cent NaOH in water.

† Color of fruit more advanced than represented by number in these cases but not sufficiently colored to be characteristic of next highest number as described in Table I.

exposed to increased quantities of ethylene. Although this result was duplicated in another test with similar fruit from this same lot, it is not known whether these results are characteristic of all ethylene-treated fruit because these tests have not been continued.

Green bananas were exposed to ethylene in concentrations from 1 in 8000 to 1 in 500 parts of air and samples were analyzed for vitamin C at

TABLE V

ASCORBIC ACID CONTENT OF BANANAS EXPOSED TO VARIOUS CONCENTRATIONS OF ETHYLENE AND HYDROGEN CYANIDE DURING STORAGE AT 19° C. (66.2° F.) (1938 FRUIT)

Conc. of gas* (parts of gas per parts of air)	Mg. ascorbic acid per 100 g. of tissue					
	Treated with ethylene for hrs.			Treated with HCN for hrs.		
	24	48	72**	24	48	72**
0	13.7	14.4	12.2	13.2	13.1	12.8
1:500	12.1	10.3	12.1	12.8	11.1	12.3
1:1000	12.5	12.3	12.4	13.1	12.2	13.0
1:2000	12.7	12.9	12.4	12.8	12.1	12.3
1:4000	12.9	12.5	13.2	13.7	12.6	11.7
1:8000	12.8	12.6	12.6	—	—	—

* Carbon dioxide absorbed by 4 per cent NaOH in water and the oxygen content was maintained between 16 and 21 per cent.

** Control fruit at this period was color 3, ethylene-treated fruit 1:500 to 1:2000 color 4, and 1:4000 to 1:8000 color 5; all HCN-treated fruit was color 4.

the end of each of three 24-hour periods. The results shown in Table V indicate again that exposure of bananas to ethylene in the absence of carbon dioxide has no detrimental effect upon the vitamin C content of the fruit.

STORAGE IN HYDROGEN CYANIDE

Considering that it might be desirable to fumigate bananas with HCN, tests were made of the effect of this gas in absence of carbon dioxide on the vitamin C content of green bananas. The results in Table V show that the HCN-treated fruit had a vitamin C content that was very little different from the untreated fruit during the course of the experiment.

DISCUSSION

The usual course of vitamin C content of the banana appears to be reasonably constant for any particular color of the fruit. Bananas used in these experiments contained about 14 mg. of ascorbic acid per 100 g. at the early green peel stage and increased slightly, then decreased with continued ripening so that they contained not less than 10 mg. at a desirable eating stage of ripeness. The values for green fruit are higher than those reported (11, 12), but repeated tests, even investigating the possibility of interference from tannins in the green fruit, showed these higher values to be easily duplicated. With further ripening beyond the brown peel stage the fruit not only lost more vitamin C but also became unfit for human consumption. The results suggest that there is a balance between vitamin C synthesis and its destruction within the banana and that there may be some relationship between the rate of respiration and the ascorbic acid content of the fruit. The typical curve for banana respiration (9) shows a low rate at the time of unloading from the boat which increases rapidly for the first few days, then decreases, at first rapidly then gradually with further ripening of the fruit. It has been suggested (14) from studies made with germinating seeds that the energy of accumulation of vitamin C is directly proportional to the energy of respiration. However, much more detailed experimental work would have to be done with bananas to determine how closely related the production of vitamin C might be to the rate of respiration. An increase in rate of respiration would indicate a general increase in metabolic processes of which sugar formation from starch would be important in the case of the banana. Sucrose, maltose, fructose, and glucose have been identified (13) in readily measurable quantities in ripening bananas. As suggested by the cultivation of the pea-embryo (15), any one of these sugars could support the formation of ascorbic acid.

Researches on other starch-containing plant parts, such as seeds (14) and potato tubers (7), have indicated the need for oxygen in the formation

of vitamin C which has not been corroborated by this work with the banana. However, this point needs further testing when uniform bananas are again available because in these experiments the bananas were not kept continuously in an absolutely oxygen-free atmosphere. Each 24 hours the containers were opened to remove samples for vitamin C analyses and the remaining fruit was exposed to the normal atmosphere with relatively high oxygen content for 20 to 30 minutes before the nitrogen atmosphere was again completely applied. It would not be expected that the banana could take up sufficient oxygen in this short period to carry it through the next 23.5 hours of the test. Also, gas analyses of the atmosphere showed less than 1 per cent of oxygen at the beginning of the storage period. However, the results show that the bananas exposed to this condition stayed green in color and maintained a uniformly high vitamin C content. Also, unlike the work with potato (7), increased percentages of oxygen caused a substantially higher level of vitamin C in the banana. No doubt this was an indirect result due to the fact that increased oxygen supply (and also ethylene where it was used) provided for an increase in the ripening process, of which vitamin C is a part, as indicated by peel color and rate of softening of the edible pulp. In all tests the vitamin C studies show that one could vary the concentration of oxygen from almost 0 to 100 per cent without showing a destruction of the vitamin C content of the fruit.

The balance between the production and destruction of vitamin C can readily be upset by the presence of carbon dioxide. The method of action of the carbon dioxide is not known although it has been observed that carbon dioxide will cause a change in the pH of the tissue and retard the rate of ripening and respiration of the fruit. However, these changes require the presence of 30 per cent or more carbon dioxide while as shown in these experiments even a small quantity of carbon dioxide produced by the fruit will cause a reduction in the vitamin C content. This was also observed in studies with asparagus (17) and other vegetables (18) but this work with the banana is different in two ways: (a) During prolonged CO_2 treatment the ascorbic acid content of the banana reaches a low value, then starts to increase. (b) If the CO_2 is removed at any time during the period of storage, the vitamin C content will increase to a value with ripening comparable with untreated fruit. This does not happen in the asparagus, which may be due to the lack of sufficient carbohydrate (2) from which the vitamin C could be replenished.

It was observed that carbon dioxide causes a change in the starch breakdown of the banana. Although the end products were not investigated, it may be that the CO_2 effect on the vitamin C content may have its inception at this point. Investigations of CO_2 effect upon the sugaring response with the potato (3, 4, 5) suggest that the controlling influence of carbon dioxide on living processes is extremely involved. Thus one cannot

support the idea that carbon dioxide is an inert gas, and fruit and vegetable handlers who store quantities of plant material in closed rooms, regardless of the temperature, should start to recognize the possibilities of its effect upon the food value of their produce.

SUMMARY

1. Green bananas (*Musa sapientum* L. var. Gros Michel), as received from the steamship, usually contained from 13 to 15 mg. of ascorbic acid per 100 g. of pulp tissue.

2. During course of ripening at 19° C. (66.2° F.) the ascorbic acid content increased slightly at the time of early development of yellow color, then fell gradually with complete yellowing to 10 to 12 mg. per 100 grams of tissue. The vitamin C content remained at this level through the period where brown flecks developed in the peel and until more than 50 per cent of the peel was brown and the pulp became soft. A further gradual reduction in vitamin C content from 10 mg. to about 7.7 mg. took place with continued browning, then blackening of the peel and development of a strong fermented flavor.

3. Variations of oxygen concentration from 0 to 100 per cent caused no detrimental effect upon the ascorbic acid content of the banana.

4. Exposure of the green banana to CO_2 resulted in a reduction of its ascorbic acid content. For example, from 3 to 5 per cent CO_2 accumulating from respiration caused a reduction of from 10 to 20 per cent in the ascorbic acid content; 8 per cent CO_2 added to the atmosphere caused a reduction of from 30 to 50 per cent; 24 per cent CO_2 caused a reduction of from 42 to 80 per cent; and 60 per cent CO_2 caused a reduction of from 66 to 85 per cent.

5. Following the reduction in ascorbic acid content of the fruit in early stages of ripening there resulted, with continuous CO_2 treatment during the courses of ripening, a gradual increase in the ascorbic acid content of the fruit until at the brown peel stage it approached the content of the untreated brown peel fruit.

6. Short-period experiments showed that while CO_2 was effective in reducing the ascorbic acid content of the green fruit, it had but little effect upon the yellow ripe fruit.

7. The removal of the bananas from CO_2 to normal atmosphere at any stage of the treatment resulted in a rapid recovery of the ascorbic acid content to a level approximating that of the untreated fruit at the same stage of ripening.

8. High concentrations of carbon dioxide caused an increase in the pH of the banana tissue of from 0.2 to 0.5 of a pH unit over that of the control fruit.

9. The starch grains of the banana treated with CO_2 decomposed into a soluble mass without showing the etching characteristic in grains during normal breakdown.

10. The presence of ethylene in concentrations from 1 to 8000 to 1 to 500 parts of air had no detrimental effect upon the ascorbic acid content of the banana.

11. A few tests with hydrogen cyanide in various concentrations indicated that this gas had no detrimental effect on the ascorbic acid content of green bananas.

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INFLUENCE OF ALUMINUM ON THE FLOWER COLOR OF HYDRANGEA MACROPHYLLA DC.

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During the past 150 years much interest has been shown in the chameleon-like character of *Hydrangea macrophylla* DC. Plants have frequently been observed to produce pink flowers one year and blue the next or vice versa. At times they may have both pink and blue flower clusters simultaneously, and even a single flower may exhibit both pink and blue areas. This alternation in coloring has stimulated much speculation and study in search of a satisfactory explanation.

Many investigators have insisted that iron absorbed from the soil was responsible for the color change; others have believed that aluminum was the effective element. The hydrogen ion concentration of the soil has also been proposed as having a direct effect upon hydrangea flower color. Still others have considered that both iron and aluminum, as well as salts of several other metals, could alter the color. It is the purpose of this paper to present experimental evidence which substantiates the view that blue flower color of *Hydrangea macrophylla* is dependent upon the presence of aluminum in the flower tissue.

HISTORICAL REVIEW

According to Siebold and Zuccarini (22, p. 106) *Hydrangea Hortensia*[2] was introduced into Japan from China. They also state that in these two countries the color was always blue because of the ferruginous substances in the clay soil. Curtis (12) gives credit to a Dr. Smith for the information that the species was introduced from China to the Royal Botanic Gardens at Kew by Sir Joseph Banks in 1790 and that it was imported at about the same time by a Mr. Slater with whom it is said to have flowered for the first time in England. Curtis (12) was the first to mention that sometimes a plant which produced red flowers one year might produce blue another though growing in the same pot. He observed this happening to a plant in the possession of the Countess of Upper Ossory in 1796.

In 1817 Neill (18, p. 122) visited a garden in Rotterdam belonging to a Dr. Daalen who is accredited with the statement that he had found the application of ashes to the roots of *Hydrangea hortensis*[2] effectual in causing the production of the fine blue color sometimes observed in the flowers of this plant. Dr. Daalen regarded the ash of Norway spruce as more effective than the common turf ash. In the same year Sprengel (23, p. 231) stated

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[2] Synonym of *Hydrangea macrophylla* DC.

that iron compounds mixed with the soil would change the red color to blue.

Schübler (20) in 1821 was probably the first to study the problem experimentally. He determined the iron content of a soil from the vicinity of Frankfurt which, according to his description, had a remarkable capacity to induce blue flowers. Since the analysis showed no greater content of iron than ordinary soil, he concluded that its effect was due to a greater content of material which he designated as carbon and humus. He suggested that these two substances absorbed the oxygen in the soil resulting in deoxidation which changed the pink color to blue. He further stated that other materials such as iron could bring about the same effect. In 1834 Schübler and Lachenmeyer (21) repeated the earlier experiments and arrived at the same conclusions. They also found iron oxide, iron sulphate, humus, and alum to be effective and explained the influence of these materials by their deoxidation hypothesis.

Also in 1821 Joseph Busch (6), gardener to the Emperor of Russia at St. Petersburgh, stated: "*Hydrangea hortensis*[2] will be turned blue by watering the young plant the summer before with allum water. Our gray colored earth under the black moor earth will have the same effect being combined with aluminous salt."

In 1846 Donald (13) tested individually the effects of phosphate of iron, alum, caustic potash, phosphate of magnesia, and carbonate of potash on single plants growing in flower pots. The plant that received alum was the only one to produce blue flowers.

Molisch (17) in 1897 tested several natural soils obtained from various parts of Europe and also many substances reported to be effective in producing blue flowers. He found that alum, aluminum sulphate, and ferrous sulphate when mixed with the soil brought about the change in color. His tests further showed that natural soils in which hydrangeas flowered blue were acid and concluded that their effectiveness was due to the consequent greater solubility of iron and aluminum. He stated that if a plant was supplied with iron in excess of its physiological need, small amounts reached the flower and reacted with the red anthocyanin to form a blue compound.

Kock, as reviewed by Molisch (17), doubted that iron influenced the production of blue flowers to any extent. In contrast to this view, Jäger, also cited by Molisch (17), believed that iron, and even alum, quite definitely brought about blue coloration.

Since Molisch (17) had observed considerable injury to his plants as a result of the treatments with aluminum sulphate, ferrous sulphate, and the like, Vouk (25) in 1908 performed experiments to determine the relative efficiency of alum and aluminum sulphate in producing blue color. Preliminary trials indicated that iron compounds had to be applied in such large quantities that the plants were seriously injured. He concluded that

potassium alum gave a more intense blue coloration than aluminum sulphate when used at the same rate.

Atkins (5) in 1923 determined the hydrogen ion concentration of pink and blue flowers and since he found no distinguishable difference, concluded that the variation in color was not due to the pigment acting as a natural indicator. He determined the iron content of pink and blue flowers and found that the pink ones contained only six-tenths as much iron as the blue. He stated that the blue color brought about by the addition of alum and aluminum sulphate to the soil was due to an increased acidity and consequent liberation of iron. He also considered that aluminum, as well as the iron, might possibly form a blue complex with anthocyanin.

Connors (11) considered that the color of hydrangeas was influenced by the hydrogen ion concentration of the soil. High concentrations tended to produce blue, while low concentrations near the neutral point produced pink. In another paper (10, p. 287) he stated: "Plants growing in soil with pH 5 or higher consistently produce blue flowers, while those in soil of pH 6.6 or lower produce pink." None of his data, however, substantiate this statement but show that blue flowers were produced when the soil was pH 5 or lower. Pink flowers were produced when the pH was 6.6 or higher.

Allen (1, 2) found that spraying pink hydrangea flowers with solutions of aluminum compounds resulted in blue coloration comparable to that observed on plants growing under natural conditions. The same effects were observed when the solutions were allowed to be absorbed through a slit in the stem. Inorganic salts of other metals did not produce a normal blue color.

In 1933 Osborn (19, p. 375) stated: "Blue color of hydrangeas is due to the presence of iron in the soil. Blue flowers may be produced by planting in a lime-free soil, notably peat, leaf mold and sand and watering freely with a weak solution of alum."

Chouard (8, 9) in 1933–1934, cited experiments in which he obtained a change in color when sulphates of iron, aluminum, chromium, copper, and uranium were supplied to the plants in soil and in sand cultures.

In 1937, Chenery (7) investigated the hydrangea problem. He presented analytical data which showed the occurrence of aluminum ions in excess of those of iron in the displaced solutions of soils that naturally produced blue flowers. Blue flowers showed a greater content of aluminum than pink ones. He also found that the extracted pigment from pink flowers changed color when solutions containing as little as 1 p.p.m. of iron or aluminum were added. The aluminum solutions changed the color to pure sky-blue, while those containing iron produced a greenish-blue tint. Solutions containing cobalt, chromium, copper, manganese, nickel, and uranium gave negative results.

In 1941 Logan, Putnam, and Cosper (15, p. 11) in their book "Science

in the garden" stated: "By now it is well known that pink hydrangeas may be turned a lovely blue by the homely device of putting rusty nails in the ground around the roots."

MATERIALS AND METHODS

Hydrangea macrophylla is a deciduous upright shrub with terminal flower clusters. The influorescence is a corymb composed of both fertile and sterile flowers. The conspicuously colored portions of the inflorescence are the petaloid sepals of the sterile flowers. The pedicels and the small and inconspicuous fertile flowers are also colored.

As a rule, the pigment is localized in a layer of cells just beneath the epidermis of the upper and lower surfaces of the sepals. The color is often more intense in the upper layer than in the lower one.

In certain varieties color sometimes appears in more than a single row of cells. These other colored cells are more or less scattered but seldom appear lower than the fourth row below the epidermis of the upper surface. The colored cells of the pedicels are subepidermal but do not form a layer. White varieties contain no colored pigment.

There is a definite sequence in which the bluing tendency of the growing medium is indicated in the floral parts. Blue anther filaments are the first sign of a tendency to produce a change. If the conditions for bluing are more favorable the color appears in the petals. A still greater bluing tendency is shown in the sepals. The blue begins to develop at the point of attachment and gradually spreads to the tips. The sequence is always followed when the color change is rapidly induced by soil treatments. The pigment is always blue from the beginning on plants grown under conditions which naturally produce clear blue color.

In addition to the two extremes in color, pink and blue, the flowers may be one of any number of intermediate hues generally classified as mauve. The mauve appearance may be produced in one of two ways: the sap of the vacuole may be uniform mauve in all the pigmented cells, or the tissue may be made up of both pink and blue cells. Since the cells are more or less distributed at random, the general aspect is intermediate between pink and blue. The actual hue depends upon the proportion of pink and blue cells. The mature flowers usually show the uniform cell coloration, while the younger ones or those in the process of change are more likely to have individual cells of both colors.

Both pink and blue flowers fade somewhat under intense light conditions or when they become old. In very old flowers a greenish pigment, presumably chlorophyll, develops which is distributed through all the parenchyma tissue of the sepals. Chlorophyll is present in very young sepals on plants growing normally. The green color persists until the sepals have grown to about their maximum size. The chlorophyll then begins to dis-

appear and the sepals take on their characteristic pink or blue color. The pink pigment is an anthocyanin classified by Lawrence *et al.* (14) as delphinidin 3-glucoside. These workers found the anthocyanins in red, blue, and violet flowers to be identical.

Under certain conditions color develops in the leaves and appears reddish or purplish. Plants which produce pink flowers show the reddish color, while on blue flowering plants it is bronzy purple.

The two clones, Mme. Chautard and Niedersachsen, were selected for most of the experiments because they grew well under greenhouse conditions and were clear pink in color. The blue phase was also bright and clear. Where other varieties were used for comparison in certain experiments, they are noted in the text.

All pH determinations were made by the quinhydrone electrode method. Reagent grade chemicals were used except where relatively large quantities were needed for soil treatments. In such cases the chemically pure grade was employed.

Aluminum was determined quantitatively by the Aluminon method (4, p. 103; 27, 28). The samples were ashed and the preliminary steps in the procedure were conducted in platinum dishes. Since it was impossible to obtain sodium hydroxide free of aluminum, this reagent was prepared from metallic sodium for the analyses. Iron was determined colorimetrically by the potassium thiocyanate method (4, p. 103). The iron and aluminum content was calculated as parts per million of dry matter, and is so expressed in the text and tables.

DESIGN OF THE EXPERIMENTS

The problem was approached in four different ways: by infiltration experiments, sand cultures, quantitative analyses, and soil treatments.

The infiltration experiments were used to test the effects of various chemical compounds on the color of mature flowers. Three techniques were employed to permit solutions of the chemicals to infiltrate the tissue. In one case the solution to be tested was sprayed on the flowers. A series of concentrations of each compound was tested to find one that was slightly injurious. This was important to make certain that the solution had actually entered the tissue. It also permitted observation of the area adjoining the injury for any slight changes in color that might occur.

The second technique was to arrange for the absorption of the solutions through the stem. Since inflorescences did not keep long enough when cut from the plant to insure a good test, a method was devised to permit absorption while the flowers remained on the plant. This was accomplished by making a slit in the stem a few inches below the inflorescence. A glass vial containing the solution was then attached to the stem so that the tongue made by the slit projected into the solution. By this method the

inflorescence kept in good condition and any effects of the chemical used could be observed over the period of the natural life of the flowers. If desired, the treatments could be started while the flowers were still in the bud stage.

The third technique was to float flowers in small beakers of the solutions to be tested. It was a less satisfactory method, except for the rapidly acting compounds, because the flowers deteriorated after two or three days.

The sand cultures offered an opportunity to control the elements supplied to the plant. They gave clear evidence of the respective effects of iron and aluminum when these elements were present or absent from the nutrient solution.

The quantitative analyses were important for comparing the iron and aluminum content of the pink and blue phases of the flower color. They also gave substantiating evidence of the influence of aluminum.

The soil treatments were significant only because they supplemented the observations from the other types of experiments. When any materials were added to the soil a number of factors were altered making it impossible to determine the direct cause of the response observed. They did, however, suggest explanations for various phenomena observed when hydrangeas were growing in the field.

RESULTS AND DISCUSSION

INFILTRATION EXPERIMENTS

Experiments with chemicals. Solutions of 30 different chemical compounds were allowed to infiltrate the tissue of mature or nearly mature flowers. This was accomplished by spraying them on the flowers, by arranging for their absorption through a slit in the stem, and by floating them in small beakers of the solution. The chemicals were used at concentrations that produced slight injury so that the color could be noted in the area surrounding the injury with assurance that the material had actually entered the tissue. The compounds tested, the concentrations used, and the color change observed adjacent to the injury are presented in Table I.

Solutions containing aluminum were the only ones that brought about a color change that was comparable to that observed in nature. Whenever aluminum was present in the solution, the area surrounding the injury was changed from pink to clear blue. With many of the aluminum compounds it was possible to find concentrations just below the point of toxicity that would induce blue coloration which appeared like that on plants growing in the field. When the artificially produced blue flowers were examined under the microscope, the blue color was found to be localized in the cells which originally contained the pink pigment.

It was observed that the iron compounds also brought about a color change but only when the concentration was high enough to be toxic. The color was not comparable to the natural condition. Instead of being bright, clear blue, it was dark, greenish-blue, sometimes varying to olive green. Under the microscope, it seemed to bear no relationship to the original pink pigment. Instead of being limited to the cells which contained the anthocyanin, it was distributed through the entire tissue. In fact, the color appeared to develop as a result of a reaction in the cell wall and cytoplasm, rather than in the vacuole.

The white flowering variety Mme. E. Moulliere was sprayed with solutions of aluminum and iron salts at slightly injurious concentrations. The typical greenish coloration was observed when the iron compounds were used, but the aluminum produced no effect except the usual indications of injury. The injury was characterized by a water-soaked lesion which later turned light brown and dried out. This experiment gave further evidence that the dark greenish color produced by iron compounds bore little or no relationship to the natural blue coloration, and that the iron was affecting cell contents other than the anthocyanin. It also suggested that aluminum influenced only the pink anthocyanin to produce the blue color.

Additional evidence to support this hypothesis was obtained from experiments with mauve colored flowers. In every case the application of aluminum brought about an intensification of the blue. It appeared that when the amount of aluminum in the pigmented cells was increased, all the pink anthocyanin changed to the blue phase.

Molisch (17) and Chenery (7) also observed a color change as a result of treatments with iron compounds, but considered it due to a reaction between the iron and the anthocyanin. According to Chenery (7) pink sepals soaked overnight in dilute solutions of iron compounds changed to a greenish-black color, while those soaked in dilute aluminum sulphate changed to sky-blue. He considered it possible that both the anthocyanin and any tanninoid substances present reacted with the iron to give the greenish-black color. The addition of iron compounds to extracted pink pigment resulted in a greenish-blue tint, while aluminum produced pure sky-blue.

Since white flowers also developed the dark greenish-blue color when sprayed with iron compounds, and since the greenish-black color was not localized in the pigmented cells of pink flowers, it would seem that the effect of iron was wholly different from that of aluminum. Even with extracted pigment (7, p. 314), the iron did not induce a color comparable to that observed under natural conditions and it is questionable whether the observation indicated any relationship to the color change in the living plant.

Experiments with aluminum and ferric ammonium citrate. Solutions of

aluminum ammonium citrate and ferric ammonium citrate could be used at much higher concentrations than the other compounds tested (Table I). Furthermore, the pH value could be adjusted without precipitation, thus eliminating any injury that might occur from an excess of hydrogen ions. Solutions of 0.5 per cent which had been adjusted to pH 6 were used extensively in experiments to observe the special effects of iron and aluminum and to secure uniform blue flowers by the spray method.

The results with aluminum ammonium citrate were very striking, but no effect on the flower color was observed for the ferric ammonium citrate. When sprayed on pink hydrangea flowers, the development of blue color could be observed in as short a time as six hours. The change usually began to appear at the edge of the sepals and gradually spread inward toward the center. One application was not sufficient to change the color completely over the entire surface of the sepals. If the treatments were started in the bud stage and repeated three or four times, it was possible to obtain uniform blue flowers comparable to those produced naturally. Figure 1 A shows an inflorescence, the blue portion of which was sprayed several times.

The hydrangea sepals were somewhat waxy, and aqueous solutions did not spread evenly over the surface. Spreaders added to the aluminum ammonium citrate solution made it possible to wet the sepals. Only a slight improvement, however, was noted in the uniformity of blue color obtained from a single application.

When the iron compound was absorbed through a slit in the stem or sprayed on plants made chlorotic by growing them in limed soil at pH 7.5, the leaves and sepals soon began to turn green and pink color developed as the flowers matured. Plants treated by the same methods with the aluminum solution did not recover from their chlorotic condition but clear blue color developed in the sepals. The aluminum was therefore effective in bringing about the blue color but did not affect the chlorosis. Iron, on the other hand, remedied the chlorotic condition, but exerted no influence on the flower color.

SAND CULTURES

To observe the effects of iron and aluminum when absorbed through the roots, hydrangea plants were grown in sand and supplied with nutrient solutions of known chemical content. Dormant field-grown plants of the

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FIGURE 1. (A, Upper left) Blue color induced by spraying portion of corymb with 0.5 per cent solution of aluminum ammonium citrate. (B, Upper right) Hydrangea flower color when the nutrient solution contained 1.34 p.p.m. of aluminum or less. (C, Center left) Mauve flower produced when the nutrient solution contained 13.48 p.p.m. of aluminum. (D, Center right) Blue flower produced when the nutrient solution contained 134.8 p.p.m. of aluminum. (E, Bottom) Plant with divided root system showing flowers of different hues on the same plant (Plant No. 3, Table V).

FIGURE 1. See legend on opposite page.

two varieties Mme. Chautard and Niedersachsen were obtained from a nurseryman. All soil was washed from the roots and the plants set in a good grade of quartz sand which had been washed several times with dilute hydrochloric acid and distilled water. New six-inch flower pots coated with hot paraffin were used for containers.

TABLE I

EFFECT OF VARIOUS CHEMICALS UPON THE COLOR OF HYDRANGEA FLOWERS. THE ORIGINAL COLOR WAS PINK

Compound	Concentration producing slight injury (per cent)	Color noted in area surrounding injury
Aluminum ammonium sulphate	0.001	Blue
Aluminum ammonium citrate	1.0	
Aluminum chloride	0.001	
Aluminum nitrate	0.001	
Aluminum potassium sulphate	0.01	
Aluminum sulphate	0.001	
Aluminum tartrate	0.05	
Ferric ammonium sulphate	0.001	Dark greenish blue
Ferric ammonium citrate	1.0	
Ferric chloride	0.001	
Ferrous sulphate	0.001	
Ammonium chloride	0.05	Pink
Barium chloride	0.05	
Beryllium chloride	0.01	
Calcium chloride	0.001	
Chromic sulphate	0.001	
Cobalt sulphate	0.001	
Copper sulphate	0.001	
Hydrochloric acid	0.001	
Lithium sulphate	0.001	
Magnesium sulphate	0.005	
Manganese sulphate	0.001	
Nickel chloride	0.001	
Potassium ammonium sulphate	0.005	
Potassium chloride	0.001	
Sodium chloride	0.005	
Stannous chloride	0.001	
Strontium chloride	0.001	
Sulphuric acid	0.001	
Zinc sulphate	0.001	

The nutrient solution was composed of the following materials: 1.50 g. ammonium nitrate, 2.50 g. calcium nitrate, 1.25 g. magnesium sulphate, 1.20 g. monobasic potassium phosphate and distilled water to make 4 liters. Just before the nutrient solution was applied to the sand, ferric or aluminum chloride was added, the amount depending upon the concentration desired for the treatments (Table II). No attempt was made to adjust the hydrogen ion concentration of the solutions and they were all extremely acid as indicated in Table II. The solutions containing the desired

quantities of iron and aluminum were applied at the rate of 250 cc. per plant at weekly intervals. The plants were watered as necessary with distilled water. Three plants of each variety were used for each treatment.

Table II gives the aluminum and iron content of the nutrient solutions applied and the flower color produced. Mauve or blue flowers developed when the aluminum content of the nutrient solution was 13.48 p.p.m. or more. In no case was there a relationship between the iron content of the nutrient solution and the blue flower color.

TABLE II

COLOR AND ALUMINUM AND IRON CONTENT OF FLOWERS FROM HYDRANGEA PLANTS GROWN IN SAND CULTURES SUPPLIED WITH NUTRIENT SOLUTIONS CONTAINING VARYING AMOUNTS OF ALUMINUM AND IRON

pH of solution	pH of sand culture 7 days after solution was applied	Iron in nutrient solution p.p.m.*	Aluminum in nutrient solution p.p.m.*	Flower color	Aluminum content of flowers p.p.m.	Iron content of flowers p.p.m.
4.8	5.6	0	0	Pink	53	196
4.0	5.5	2.79	0	Pink	71	201
3.2	5.3	27.9	0	Pink	40	368
1.6	4.1	279.0	0	Pink	86	943
1.0	3.5	2790.0	0	—**	—	—
3.7	5.5	2.79	1.348	Pink***	147	190
3.3	5.1	2.79	13.48	Mauve	235	231
2.6	5.1	2.79	134.8	Blue	1269	256
2.4	4.0	2.79	1348.0	Blue	4572	278
4.1	5.6	0	1.348	Pink***	187	216
3.3	5.3	0	13.48	Mauve	203	222
2.6	5.0	0	134.8	Blue	981	261
2.4	4.1	0	1348.0	Blue	4200	248

* Actual aluminum (Al) and iron (Fe) calculated from the amount of aluminum and ferric chloride added.

** Plants died.

*** Anther filaments blue.

Within certain limits the intensity of the blue was proportional to the aluminum content. A concentration of 1.34 p.p.m. was not enough to produce blue color within the period of the experiment except in the anther filaments (Fig. 1 B). Mauve or intermediate hues developed when the nutrient solution contained 13.48 p.p.m. of aluminum (Fig. 1 C), while 134.8 p.p.m. induced a clear blue coloration (Fig. 1 D).

This experiment gave additional evidence that aluminum and not iron was responsible for producing blue color in hydrangeas. While there seemed to be a correlation between the amount of aluminum in the nutrient solution and the hue of blue produced, it should not be concluded that 13.48 p.p.m. of aluminum will always produce mauve flowers. The time element was undoubtedly a very important factor in this experiment. It is quite likely that had the plants been grown from cuttings under the same

conditions, or the treatments extended over a longer period of time, 13.48 p.p.m. would have induced clear blue color.

Only a few of the plants in the experiment showed iron deficiency symptoms when iron was omitted from the nutrient solution. It was suspected that the plants were obtaining iron from impurities in the sand or else enough was contained in the dormant plants to sustain normal foliage color during the forcing period. A potassium thiocyanate test for iron in a water extract of the sand was negative, but a dilute hydrochloric acid extract showed a positive test. Because of the low pH values of most of the nutrient solutions, it seemed quite likely that the plants were obtaining considerable iron from the culture medium.

In order to differentiate clearly the individual effects of iron and aluminum, another sand culture experiment was conducted using sand that had been thoroughly washed with aqua regia until a hydrochloric acid extract showed no trace of iron. The plants were also especially prepared by growing them from cuttings in soil at pH 6.8 to 7 in order to keep them as low in iron and aluminum as possible and still not interfere with their growth. After flower buds had formed and the rest period was completed, the soil was entirely removed from the roots and the plants set in the purified sand.

Glazed crocks of one liter capacity with a drainage hole in the bottom were used for containers. A constant drip system was arranged and adjusted to supply one liter of solution per day to each container. Three plants of the variety Niedersachsen were used for each treatment.

The nutrient solution was the same as used in the earlier experiment except that 15 cc. of 0.5 M. sodium citrate solution was added to each liter to keep the iron and aluminum soluble. After the ferric or aluminum chloride or both were added, each solution was adjusted to pH 5 by the addition of sodium hydroxide or hydrochloric acid as needed. Since in the previous experiment 2.79 p.p.m. of iron gave good growth and 134.8 p.p.m. of aluminum produced blue flowers, these amounts were selected for the treatments. The treatments given and the resulting effect on flower color, condition of foliage, and growth are found in Table III.

TABLE III

FLOWER COLOR, CONDITION OF FOLIAGE, AND GROWTH OF HYDRANGEA PLANTS IN SAND CULTURES. IRON-FREE SAND WAS USED AND THE NUTRIENT SOLUTIONS WERE ADJUSTED TO pH 5

Iron content p.p.m.	Aluminum content p.p.m.	Condition of foliage	Growth	Flower color
0	0	Very chlorotic	Poor	Pink
2.79	0	Normal	Good	Pink
0	134.8	Very chlorotic	Poor	Blue
2.79	134.8	Normal	Excellent	Blue

The plants grown with nutrient solutions containing no iron produced pink flowers, but the foliage and buds were very chlorotic. When iron alone was added, the flowers were also pink, but the chlorosis was entirely prevented. Aluminum added to the nutrient solution without iron resulted in blue flowers on very chlorotic plants. The flower color was like that shown in Figure 1 D. When both iron and aluminum were added, the flowers were blue and the foliage normal green. In fact, these plants were noticeably larger and more vigorous than in any of the other treatments.

The results of this experiment gave strong evidence that aluminum rather than iron was responsible for inducing blue flowers. No evidence came to light to suggest that iron influenced the flower color except in so far as it affected the life of the plant.

Molisch (17), Atkins (5), and others concluded that iron caused the blue color which is at variance with the results of these experiments. The present findings are in agreement with the work of Allen (1) and Chenery (7) who gave evidence to support the view that blue coloration was the result of the absorption of aluminum. The experiments of Chouard (8, 9) reporting that iron, chromium, aluminum, copper, and cobalt could produce the bluing-agent were not supported by data and no evidence was obtained in the present work to substantiate his claims except in the case of aluminum.

QUANTITATIVE ANALYSES

Aluminum and iron content of flowers from plants grown in soil. Analyses of hydrangea flowers showed clearly that the blue ones contained more aluminum than the pink and supplemented other evidence that blue color was dependent upon the presence of aluminum in the tissue of the flower. The mean aluminum content of three blue inflorescences from a single plant was found to be 821 p.p.m. Similar determinations of three pink flower clusters from one plant gave 166 p.p.m. On the basis of these data the blue contained 4.9 times as much aluminum as the pink.

A ratio of 15.7 was reported by Chenery (7) which was approximately three times that obtained in these analyses. The explanation probably lies in the fact that he used, for the most part, flowers collected from plants growing in the open. In the present experiments this method was found to yield very variable data. The aluminum content varied widely, some samples containing as much as 4000 p.p.m.

The most important factor affecting the aluminum content was the hydrogen ion concentration of the soil in which the plants were growing; the lower the pH values, the higher the aluminum content. Table IV includes data on the aluminum content of flowers from plants grown from cuttings in soils of known pH values. While there was no apparent difference between the blue color of flowers grown at pH 5.13 and those grown at pH 4.56, the difference in aluminum content was 1478 p.p.m.

Where aluminum sulphate had been added to acidify the soil, the flowers showed a high aluminum content even though the difference in pH value was not great. Flowers from untreated soil at pH 5.1 contained 897 p.p.m. of aluminum. When a little aluminum sulphate was added and the pH lowered to 4.85, the aluminum content more than doubled or increased to 1906 p.p.m. (Table IV).

By comparing the aluminum content of the blue flowers grown at pH 4.56 with that of pink ones grown at pH 6.92 (Table IV), a ratio of 33.9 was

TABLE IV

COLOR AND ALUMINUM AND IRON CONTENT OF HYDRANGEA FLOWERS GROWN FROM CUTTINGS IN SOILS OF VARYING pH VALUES

Average pH value	Flower color	Aluminum content p.p.m.	Iron content p.p.m.
4.56*	Blue	2375	386
4.65*	Blue	2482	516
4.74*	Blue	2100	310
4.85*	Blue	1906	502
5.13	Blue	897	430
5.50	Blue tinged pink	338	300
5.75	Blue tinged pink	270	264
6.01	Pinkish blue	241	232
6.25	Mauve	289	219
6.44	Bluish pink	187	222
6.51	Pink tinged blue	214	229
6.70	Pink except anther filaments	201	237
6.89	Pink	180	235
6.92	Pink	70	267
7.02	Pink	187	182
7.16	Pink	193	190
7.36	Pink	217	123

* Aluminum sulphate added to lower the pH value of the soil.

obtained. The exact ratio was therefore considered of little significance in the problem because it varied widely according to the source of the data used for its calculation.

On the basis of the data presented in Table IV an aluminum content of approximately 250 p.p.m. in the flowers was considered minimum for the production of a distinctly bluish hue on plants growing in soil. Flowers with less than 150 p.p.m. showed no trace of blue. Flowers containing from 150 to 250 p.p.m. normally showed intermediate stages between pink and blue. Bright blue flowers growing in the field usually contained from 800 to 900 p.p.m. of aluminum.

The iron content showed less relationship to the flower color than did the content of aluminum. In general, the amount of iron in bright blue flowers was considerably higher than that of pure pink ones. Like the aluminum content, the amount of iron was affected by the hydrogen ion

concentration of the soil in which the plants were grown although the extremes were not as great (Table IV).

Atkins (5) also found that blue flowers contained more iron than pink ones and concluded that iron was responsible for the color change. While his observations were in close agreement with those of this study, his interpretation was quite different. The iron content of blue flowers was higher than the pink because the conditions favorable for the absorption of aluminum were also conducive to the absorption of iron from the growing medium.

Aluminum and iron content of flowers from sand cultures. Flowers from the sand culture experiments were analyzed for aluminum and iron and the data are included in Table II. Iron and aluminum varied greatly in the flowers according to the amount of these elements added to the nutrient solution.

There was a direct relationship between the aluminum content and the blue color, but no such relationship existed between the amount of iron and the color. Instances were observed where considerably more iron was found in pink flowers than in blue. Certain pink flowers contained 943 p.p.m. of iron, while certain blue ones contained only 261 p.p.m. (Table II). When no iron was supplied in the nutrient solution, the iron content of the flowers varied from 190 p.p.m. to 278 p.p.m. The greatest amount of aluminum obtained in flowers when this element was omitted from the nutrient solution was 86 p.p.m. These data seemed to indicate that a certain amount of aluminum and iron was obtained from impurities in the sand or was present in the plants at the time they were transferred from the soil.

SOIL TREATMENTS

Relationship between soil pH value and blue coloration. Early in the 19th century the color change in hydrangeas was first considered to be associated with soil conditions. Since that time, many different materials have been reported to affect flower color when applied to the soil. Changing the chemical composition of soil by the addition of inorganic compounds usually alters the hydrogen ion concentration, which in turn affects the solubility of certain elements, particularly aluminum (16).

Several experiments were made with hydrangeas grown in flower pots to determine the relationship between flower color and the hydrogen ion concentration of the soil. Varying amounts of aluminum sulphate and calcium carbonate were thoroughly mixed with naturally acid soil (pH 5.1) to produce a series of pH values. The mixtures were stored in a moist condition for about two months before beginning the experiment to allow for complete adjustment of the hydrogen ion concentration. Rooted cuttings were potted in the soil mixtures and a pH determination made for

each. Other pH readings were made at monthly intervals during the growing seasons. There was little change in pH value during the course of the experiment, but any pots which showed a variation of more than 0.5 of a pH unit were discarded. A minimum of five plants was used in each treatment.

Only blue flowers were produced when the pH value was below 5.5 (Table IV). From pH 5.5 to 6.25 the flowers showed a definite pink hue which increased in prominence as the pH value increased. At pH 6.25 the color was considered intermediate between blue and pink. Between pH 6.25 and 6.70 the flowers were pink, but a blue hue was in evidence. From pH 6.7 to 7.36 the plants produced only clear pink flowers.

These data are in agreement with those reported by Wiggin and Gourley (26) except that they did not distinguish the intermediate hues. According to these workers, the flowers produced in soil plots at pH 4.0 to 5.0 were all blue; from pH 5.0 to 6.0 there were occasional blue flowers; from pH 6.0 to 8.0 the flowers were always pink.

Atkins (5) found that plants growing in soils of pH 5.75 and 6.0 produced blue flowers. Pink, intermediate, or blue flowers were produced in soils of pH 5.9, 6.2, and 7.3. Where the pH value was 7.5, 7.6, and 8.0 all flowers were pink.

Atkins' limited determinations were made by testing the pH value of the soil in the proximity of plants growing in the field. To test the reliability of this method, a large number of pH determinations were made on soil samples collected from around plants of various hues. Samples from near bright blue flowering plants ranged from pH 4.9 to 7.2, while those from around plants bearing pink flowers varied from pH 5.2 to 7.4. The fact that hydrangeas are commmonly grown in flower pots and transplanted to the open ground seemed to explain the variability since it was observed that the color of the flowers was frequently influenced by the potting soil used in the early growth of the plants.

The results were the opposite of those reported by Connors (10, p. 287) who stated: "Plants growing in soils with pH 5.0 or higher, consistently produce blue flowers, while those in a soil of pH 6.6 or lower produce pink." It is quite likely that Connors' statement was a misprint or he considered pH to be synonymous with hydrogen ion concentration, since this paper and another one (11) showed that blue color was produced only under acid soil conditions.

The fact that blue hydrangeas are produced normally only in acid soil is not evidence that the hydrogen ion concentration directly influences the color. In the sand culture experiment (Table II) the pH value of all the solutions was below that which induced blue flowers in soil. If the hydrogen ion concentration had been a direct factor in the production of blue flowers, the fact would have undoubtedly appeared in that experiment.

The solubility of aluminum in the soil has been shown by Magistad (16) to depend upon the hydrogen ion concentration. Turner (24) found that aluminum was invariably absent from filtrates obtained from soils with pH values between 7.5 and 6.7, but was always present when the pH value was below 5.1. Chenery (7) also determined a definitely higher free aluminum content in the more acid soils and recorded 6.7 p.p.m. of ionic aluminum at pH 4, and 0.01 p.p.m. at pH 6.3.

The increased solubility of aluminum at low pH values explains the development of blue color in acid soils. The hydrogen ion concentration should be considered a factor in the development of blue or pink color only in so far as it affects the solubility of aluminum in the soil.

Blue flowers in alkaline soil. Due to its amphoteric character, aluminum is known to be soluble at pH values higher than 7.2 and the amount of aluminum present in soil at pH 8 is about the same as at pH 5 (24). In acid solutions the aluminum ion is a cation, while in alkaline solutions it forms the aluminate radical (AlO_3). It might be expected, therefore, that blue flowers could be produced in strongly alkaline soil. To determine this point, cuttings were potted in soil made alkaline with calcium hydroxide to pH 7.5 or above and to which aluminum phosphate had been added to insure an abundance of aluminum. The plants became extremely chlorotic and their growth was very poor. Many of them died during the first growing season and most of those that lived failed to develop flower buds.

One of the plants that reached the blooming state produced blue flowers but they were not as brilliant and clear as those grown in acid soil. The poor color may have been due in part to the unhealthy condition of the plant. Other plants produced pinkish-blue flowers indicating that some aluminum was being absorbed.

In another experiment, plants growing in alkaline soil were watered several times with a one per cent solution of aluminum ammonium citrate. No clear blue flowers were produced, but they all showed a definite bluish hue, while the untreated plants were pure pink.

It was quite possible that by this treatment the roots were badly injured either by the high alkalinity of the soil or the chemical applied. Injury to the roots would undoubtedly facilitate diffusion or mass flow of the soil solution into the roots. This idea was borne out in other tests where it was found that a much more rapid bluing could be accomplished if a pencil was pushed several times through the soil mass just prior to applying the solution. This treatment facilitated a more even penetration of the solution to all parts of the root system, but also broke off many small rootlets which permitted easy entrance of the solution as applied.

The experiments with alkaline soil indicated that blue flowers may be produced under high pH conditions. If it were possible for hydrangeas to grow well in extremely alkaline soil, it seems quite probable that the color

would be blue if the soil naturally contained an abundance of aluminum.

Accumulation of aluminum within the plant. During the progress of the experiments, considerable evidence accumulated to suggest that aluminum might be stored within the plant. If hydrangeas were grown in an acid soil and transferred to an alkaline one, they invariably produced blue or mauve flowers unless the transfer was made early in the life of the plant, even though the original soil was thoroughly washed from the roots. Cuttings, on the other hand, made from blue flowering plants produced pink flowers when grown in neutral or alkaline soil.

Plants grown in soil near the neutral point produced blue or mauve colored flowers if transferred to acid soil or to soil treated with aluminum sulphate as late as three weeks before flowering. It has been observed by commercial growers that plants produced in acid soil to insure vigorous growth often show bluish hues when forced in soil at pH values well up in the pink-producing range.

Allen (3) attempted to control the color of hydrangeas by applying various substances in solution to potted plants during the forcing period. Five weekly applications of a 2.5 per cent solution of aluminum sulphate to plants growing in five-inch flower pots were sufficient to produce clear blue flowers.

These observations indicate that aluminum can be accumulated within the plant to produce blue flowers when transferred to alkaline soil. On the other hand, if the plants are transferred to acid soil or treated with aluminum sulphate solution they absorb aluminum rapidly and produce blue flowers.

Flowers of different colors on the same plant. Single plants are often observed with inflorescences of more than one hue. Sometimes the flowers of a single inflorescence vary from clear blue to clear pink. Connors (11) suggested this phenomenon was due to a lack of uniformity in the soil.

An experiment was conducted to determine whether or not plants could be induced to develop vari-colored flowers under experimental conditions and to test Connors' hypothesis. Two holes were drilled in the bottoms of three-gallon crocks and a vertical glass partition was cemented in the center to make two water-tight compartments with a drainage hole in each. Single node cuttings were rooted in sand and when both buds had started to develop, a slit was made from the base to one-half inch below the node to divide the root system into two parts as nearly equal in size as possible. The cuttings were then placed so that the glass plate separated the two halves of the root system. The two compartments were then filled with various soil mixtures. The treatments and the color of the inflorescences produced are included in Table V.

When the plants bloomed, inflorescences of different colors appeared on the same plant according to the soil treatments given the two halves of

the root system. In the case of plant No. 1 (Table V) ferrous sulphate had been mixed with the soil in compartment A, making it acid (pH 4.5 to 5.5). Lime had been added to the soil in compartment B and the pH value ranged from 6.4 to 6.9. The flower produced over the limed soil was pure pink in color with no trace of blue, while the flowers on the opposite side were clear blue.

TABLE V

FLOWER COLOR OF PLANTS GROWN WITH DIVIDED ROOT SYSTEMS

Plant No.	Compartment	Soil treatment	pH at outset	pH at flowering	No. and color of inflorescences
1	A	Ferrous sulphate	4.5	5.5	3 Blue
	B	Lime	6.4	6.9	1 Pink
2	A	None	4.6	5.6	3 Blue
	B	Lime	6.2	7.1	1 Blue 1 Pink to mauve
3	A	Potassium alum	4.4	5.6	1 Blue
	B	Lime	6.2	6.9	2 Blue 1 Blue to Pink 1 Pink
4	A	Magnesium sulphate	4.7	5.8	2 Blue
	B	Lime	6.1	6.9	2 Pink to mauve
5	A	Aluminum sulphate	4.1	4.8	1 Blue
	B	Lime	6.5	6.9	2 Blue 1 Blue to mauve

A slightly different response was observed for plant No. 2 where compartment A contained naturally acid soil and compartment B limed soil. The flowers above the acid side were clear blue, but one inflorescence over the limed soil varied from mauve to pink. A second inflorescence on the same side was clear blue. Plant No. 3, shown in Figure 1 E, as well as plants No. 4 and 5, were similar in character in that they also produced blue or vari-colored blooms above the limed soil compartment.

If no other factors were operating, all of the flowers over the limed soil should have been pink, while those over the acid soil should have been blue. Since this was not the case with plants No. 2, 3, 4 and 5, it appeared that aluminum was being translocated from the root system in the acid soil to the flowers on the opposite side of the plant.

To determine whether or not there was a vascular connection between the roots in acid soil and the blue flowers, the plants were removed from the crocks and the roots washed and trimmed to about three inches in

length. The roots which had been growing in an acid medium were placed in a 0.1 per cent solution of the stain, Light Green SF Yellowish. The other half of the root system was placed in a solution of the stain, Amaranth, of the same concentration. The plants were allowed to absorb the dyes until the respective colors could be seen in the sepals. The cortex and phloem were then removed to expose the xylem and show the path of the dyes.

In plant No. 1 (Table V) no trace of the green dye could be seen on the opposite side of the plant and the exposed xylem showed no vascular connection between the roots growing in compartment A and the flowers over compartment B. There was, however, a vascular connection between the roots from the limed soil (compartment B) and the flowers on the opposite side (compartment A).

In plant No. 3 (Fig. 1E) the branch on the extreme right had a vascular connection with only the roots in the limed soil (compartment B). Apparently the base of the cutting had not been slit in the exact middle or new tissue had regenerated at the upper part of the slit above the glass partition. Other flowers on this same side were connected with the roots in the acid soil which accounted for the development of the blue flowers.

One corymb varied from bright blue through mauve to pink (Fig. 1 E). There was a direct relationship between the amount of xylem carrying the green dye and the flower color; the greater the amount of vascular tissue connecting the flowers with the roots in acid soil, the clearer blue the color.

Since the green stain reached all the blue flowers, it was assumed that aluminum absorbed by the roots could do likewise. This assumption is borne out by the data of Chenery (7) which show a greater amount of aluminum in the blue portion of a vari-colored inflorescence than in the pink portion.

These experiments demonstrate that flowers of different hues can be induced on an individual plant by treating parts of the root system differently. They support the suggestion (11) that plants growing in the field produce vari-colored flowers because of a lack of uniformity in the soil. Roots growing in pockets of acid soil may absorb enough aluminum to induce blue color in the flowers with which they have vascular connections.

Varietal differences. Commercial growers have observed that certain varieties of hydrangeas such as Mme. Chautard, Niedersachsen, General de Vibraye, Sensation, and Gert Glahn produce blue flowers readily when the plants are grown in acid soil. The blue phase is difficult to obtain in varieties such as Mein Liebling, Elmar, Nixe, Rochambeau, and others.

About 75 commercial varieties of hydrangeas were grown in neutral soil (pH 7) and soil made acid (pH 5) by the addition of aluminum sulphate. Some of the varieties produced a bright clear blue, while others produced mauve and unattractive hues intermediate between pink and blue. In Mein Liebling, the color was practically pure pink with only a

slight tinge of blue. It was possible to obtain clear blue color in all the varieties when aluminum ammonium citrate was sprayed on the flowers or absorbed through a slit in the stem.

SUMMARY

1. Experiments conducted demonstrate conclusively that the blue flower color of *Hydrangea macrophylla* is due to the presence of aluminum in the flower tissue. Aluminum compounds consistently induced a change in color from pink to blue when allowed to infiltrate the tissue of mature flowers. Iron compounds produced a dark greenish-blue color that was not comparable to the natural blue coloration.

2. Iron compounds allowed to infiltrate into chlorotic plants induced chlorophyll development in the leaves and buds, but did not affect the color of the flowers. Aluminum compounds induced blue color but did not remedy the chlorotic condition.

3. The flower color of plants grown in sand cultures was closely related to the aluminum content of the nutrient solution. A nutrient solution containing 13.48 p.p.m. of aluminum produced mauve colored flowers, while 134.8 p.p.m. induced clear blue flowers. With especially prepared plants and purified sand, the individual effects of iron and aluminum were shown. Iron in the nutrient solution prevented chlorosis in the leaves and flower buds, but did not affect flower color. Aluminum induced blue flower color, but did not prevent chlorosis.

4. The aluminum content of blue flowers was higher than that of pink ones. Blue flowers from plants grown in sand cultures had an aluminum content of more than 250 p.p.m.; pink flowers contained less than 150 p.p.m., while mauve flowers varied from approximately 150 to 250 p.p.m. Bright blue flowers from plants growing in the field usually contained 800 to 900 p.p.m. of aluminum, although the amount varied widely depending upon the conditions under which the plants were growing. Aluminum compounds added to the soil increased the aluminum content of the flowers.

5. The iron content showed less relationship to the flower color than did the aluminum. Blue flowers from plants growing in soil had a higher iron content than pink ones, but in sand cultures where the amount of iron in the nutrient solution was controlled, some of the blue flowers contained less iron than the pink.

6. Flowers produced on plants grown from cuttings in soil at pH 5.5 or below were blue. Between pH 5.5 and 6.25 the flowers were intermediate between pink and blue. Above pH 6.7 the flowers normally showed no trace of blue. Blue flowers were produced on plants growing in alkaline soil at pH 7.5 or above. There was evidence that aluminum could be accumulated in the tissue and bring about mauve or blue color when the plants were transferred to neutral or alkaline soil.

7. Single plants were caused to produce flowers varying from clear blue to clear pink by dividing the root system and growing the two halves in different types of soil. Wherever there was a vascular connection between the roots growing in acid or aluminum sulphate treated soil, the flowers were blue.

8. Some varieties failed to produce clear blue flowers in soil made acid by the addition of aluminum sulphate. Flowers of the same varieties turned blue when aluminum compounds were sprayed on mature sepals or absorbed through a slit in the stem.

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FURTHER TESTS ON PLANT PRODUCTS FOR INSECTICIDAL PROPERTIES

Albert Hartzell

The present paper is a continuation of work previously reported (12) on plant products possessing insecticidal properties. Selected references appearing since the publication of our previous paper are cited here without attempting to cover the voluminous literature on this subject, as the present investigation was designed to find other plant products that would supplement or even replace our diminishing supplies of these materials due to the war. A review of the literature of insecticidal materials of vegetable origin including nicotine, anabasine, hellebore, rotenone-containing plants, pyrethrum, quassia, and various plant oils has been made by Holman (15).

The kernels of *Thevetia neriifolia* Juss. were reported by Cherian and Ramachandran (4) to be a potent insecticide.

Preliminary tests by Apple and Howard (2) with flours made with the meat hulls of nuts of California buckeye (*Aesculus californica*) showed toxicity to the larvae and adults of Mexican bean beetle.

LaForge and others (17) extracted a toxic substance from the bark of southern prickly ash (*Zanthoxylum clava-herculis* L.) that is toxic to houseflies and possesses a toxic action similar to pyrethrum.

Jervine and pseudojervine were found to be the predominant alkaloids in American hellebore (*Veratrum verde* Aiton) by Seiferle, Johns, and Richardson (23). Both alkaloids are toxic to the American roach (*Periplaneta americana* L.).

The seed of *Millettia pachycarpa* Benth. was shown to be toxic to the housefly (*Musca domestica* Linn.), cabbage worm (*Pieris rapae* Linn.), China-grass butterfly (*Pareva vesta* Fabr.), and silkworm (*Bombyx mori* Linn.) by Chiu and others (6). The active principle is both a contact and a stomach poison. In a later article these authors (5) reported that this plant product is as toxic as rotenone.

Sesamin and related compounds were reported by Haller and others (8) to have a synergistic effect on pyrethrum when applied to houseflies. The nature of the substituents on the benzene ring is believed by them to be the determining factor in the synergistic action of this class of compounds.

Aerosols prepared from dichlorodifluoromethane solution of pyrethrins with sesame oil as a synergist were found by Billings and others (3) to be effective against the cheese skipper (*Piophila casei*).

Thanite, the thiocyanoacetate of secondary terpene alcohols, was found to be an activator of fly sprays (16).

Higbee (14) lists insecticidal plants other than pyrethrum, *Nicotiana*,

and rotenone-containing products that are grown in the Americas as follows: citronella; *Piper aduncum*, used to repel ants in Haiti; *Picramnia pentandra; Vetiveria zizanioides*, the roots of which are used to ward off clothes moths and to kill plant lice and bed bugs; cucaracha herb (*Haplophyton cimicidum*) from Mexico and Central America, used to destroy head lice and cockroaches. West Indian quassia boxes, he reports, are used for the storage of clothing to protect it from moths. Fly paper can be made of blotting paper soaked in a sugared extract of that wood. Other insecticidal plants listed by him are: *Delphinium; Petiveria alliacea; Rosmarinus officinalis; Pimenta acris*, from which bay rum is obtained; *Eucalyptus;* and *Datura arborea*, used in Cuba to repel giant ants.

The distribution and chemical constituents of 164 varieties and species of plants having insecticidal and piscicidal properties are listed by Chopra and others (7).

An extract of the non-volatile residue of the fruit of the Amur corktree was found by Schechter and Haller (22) to be toxic to mosquito and codling moth larvae and to houseflies. The unsaponifiable portion of the oil is very toxic to houseflies in acetone but not in high boiling kerosene.

The Chinese yam bean was reported by Hansberry (9, 10, 11) and others (19) to contain an active principle effective in the control of the pea aphis, Mexican bean beetle, and other pests, particularly when the ground seeds were used as a dust with talc.

According to Pollacci and Gallotti (20), *Solanum nigrum* contains an active principle, solanin, which is reported an excellent agricultural insecticide when used in a solution at a concentration of 0.05 per cent.

The powdered seed of sabadilla has long been used as an insecticide against lice and roaches. Allen and Dicke (1) report that a sabadilla spray is effective in the control of houseflies.

MATERIALS AND METHODS

The crude botanical drugs were furnished through the courtesy of S. B. Penick & Company, New York, N. Y.

The following species of plants from which vegetable products were obtained were grown in the Institute herb and flower gardens: azaleamum, bene, betony, costmary, dill, geranium, gerbera, hyssop, lavender, lemon geranium, lovage, marigold, mimulus, mugwort, nasturtium, old man, parsley, peppermint, poppy, sage, spearmint, sweet basil, sweet woodruff, and wormwood.

Butter and eggs, celandine, euphorbia herb, flowering dogwood, gourd, milkweed, sweet fern, and *Trichosanthes* were grown in the Boyce Thompson Arboretum or obtained locally. Scientific names for plants grown by the Institute appear in Table I.

Among spices and condiments, allspice, cinnamon, cloves, black pepper,

and cayenne pepper were obtained from the Atlantic and Pacific Tea Co. Black pepper was also obtained from S. B. Penick & Company.

METHODS OF EXTRACTION

The dried plant products were ground into a fine powder in an electric grinder (Hobart food cutter). The powdered plant extracts were placed in extraction thimbles, weighed on an analytical balance, and extracted with acetone in a Soxhlet extractor overnight (16 hours). For the strongest concentration of acetone extract tested, a quantity of extract equivalent to 4 g. of dry material was made up to one liter. With water extractions an extract of 8 g. was made up to one liter. The range of concentrations tested, based on dry weight of powder at the start, was 16 to 4000 p.p.m. with acetone extract, and from 500 to 8000 p.p.m. with water extracts.

BIOLOGICAL TESTS

Tests were conducted according to methods previously described (12). Mosquito eggs[1] were shipped weekly from Orlando, Florida, to Yonkers, New York, via air mail. When the larvae hatched, they were fed yeast (575 mg. to 4 l. of water) and blood albumen (500 mg. to 4 l. of water) on alternate days. Larvae from five to six days old were used for testing. Test tubes (25 cc. capacity) containing 10 larvae each in the solution to be tested and controls in tap water alone, and acetone and water were placed in an oven at $29° \pm 1°$ C. overnight (16 hours). All tests were run in duplicate and in at least four different concentrations differing by a dose ratio of 2. Acetone controls consisted of 1.5 cc. of acetone to one liter of water. This concentration was found to be non-toxic to the mosquito larvae. If one or more larvae were found dead in the checks, the tests were repeated.

The tests to determine whether a material possessed promise as a contact insecticide were made with *Aphis rumicis* L. on nasturtium as described previously (12).

To determine whether a substance possessed properties of a stomach poison, the spray material was made up with a wetting agent (0.5 per cent Penetrol) and applied with an atomizer on bean plants (*Phaseolus vulgaris* L. var. *humilis* Alef.). Mexican bean beetle (*Epilachna varivestis* Muls.) larvae and adults were colonized on sprayed plants after the foliage had dried. The control plants were sprayed with the wetting agent and allowed to dry before being colonized. The tests were conducted under greenhouse conditions in cages specially designed for that purpose. The cage (Fig. 1) was constructed with wooden frames ($12 \times 12 \times 12$ in.), and with glass panes 2-1/2 in. wide set in the frames 1/8 inch apart. The floor of the cage was made of Flexboard. A vertical sliding door afforded access for intro-

[1] Obtained through the courtesy of C. H. Bradley.

ducing potted bean plants and insects. The cage permitted sufficient circulation of air so as to prevent "steaming" of the glass by the respiration of the plants. When testing larvae the cages were covered with cheesecloth.

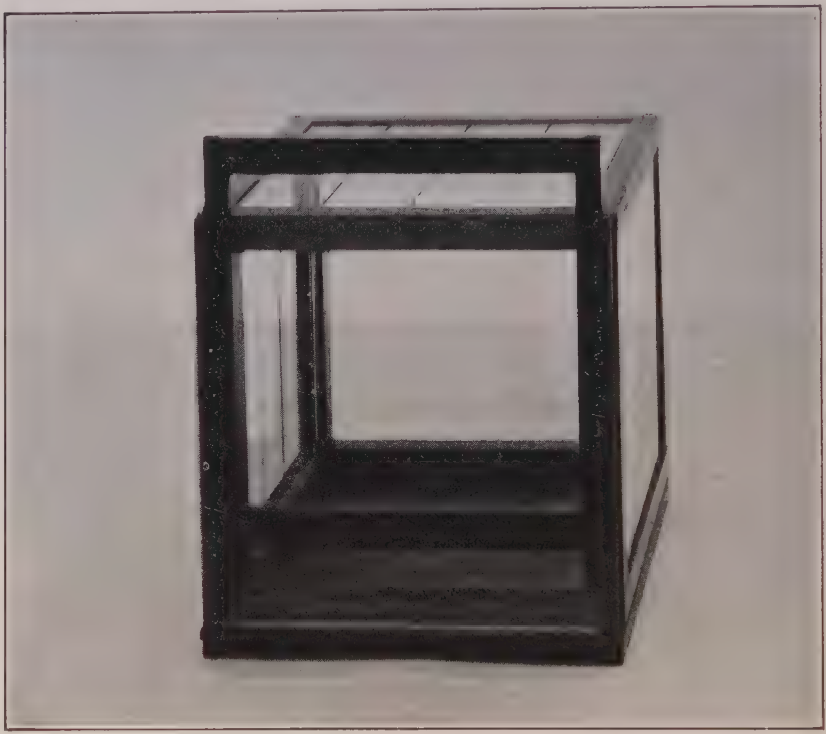

FIGURE 1. Cage used for testing plant insecticides on Mexican bean beetle adults (*Epilachna varivestis*) on potted bean plant. Glass panes set 1/8 in. apart in frames to allow for circulation of air and to prevent "steaming" of the glass.

RESULTS

TOXICITY OF EXTRACTS TO MOSQUITO LARVAE

The median lethal dose (LD50) of acetone extracts of plant products to mosquito larvae (*Culex quinquefasciatus* Say) are listed in Table I. The values were obtained by interpolation in all cases. Water extracts were also made of all plant products listed in this paper. Acetone proved to be the better solvent. The LD50 values of water extracts of the three most toxic plant products tested follow: licorice 8000 p.p.m., red cedar chips (*Juniperus virginiana* L.) 3500 p.p.m., and sesame seed 8000 p.p.m.

In addition to the plant products listed in Table I, ephedrine, an alkaloid (U.S.P.) of *Ephedra* sp., loganin, a glycoside of *Strychnos nux-*

TABLE I
ACETONE EXTRACTS OF PLANT PRODUCTS TESTED ON MOSQUITO LARVAE
(CULEX QUINQUEFASCIATUS)

Common or trade name	Scientific name	Plant part	LD50 p.p.m.
A. Plant products with LD50 values less than 1000 p.p.m.			
Anise seed, Spanish, N.F.*‡	*Pimpinella anisum*	Seeds	470
Cloves		Flower buds	780
Costmary	*Chrysanthemum balsamita* L.	Leaves & stems	590
Cubeb berries, stemless, N.F.*	*Piper cubeba*	Berries	24
Cucumber, Henderson**		Seeds	520
Cumin seed*	*Cuminum* sp.	Seeds	960
Elder flowers, Bright, N.F.*	*Sambucus canadensis* or *nigra*	Flowers	300
Fennel seed, French*	*Foeniculum vulgare*	Seeds	540
Gourd	*Cucurbita pepo* L. var. *ovifera* Bailey	Seeds	580
Jalap root, 15% N.F.*	*Exogonium jalapa*	Root	380
Koelreuteria†	*Koelreuteria paniculata* Laxm.	Seeds & leaves	245
Larkspur seed, N.F.*	*Delphinium ajacis*	Seeds	540
Melon, Honey dew**		Seeds	520
Pepper, Black*	*Piper nigrum*	Dried berries	29
Poppy	*Papaver* sp.	Flowers & stems	440
Poppy seed, Dutch*		Seeds	700
Prickly ash bark, N.F., Southern*	*Xanthoxylum clava herculis*	Bark	540
Pumpkin, Striped Cushaw††		Seeds	550
Sandalwood, N.F. (chips), select*	*Santalum album*	Wood	900
Squash, Blue Hubbard††		Seeds	700
Squash, Connecticut Straightneck††		Seeds	460
Squash, Golden Summer Crookneck††		Seeds	450
Squash, Winter††		Seeds	700
Watermelon seed*	*Citrullus vulgaris*	Seeds	560
B. Plant products with LD50 values from 1000 to 4000 p.p.m.			
Agrimony herb*	*Agrimonia eupatoria*	Whole plant	3100
Althea root, U.S.P. (peeled)*	*Althaea officinalis*	Root	3000
Azaleamum	*Chrysanthemum* sp.	Leaves, stems & flowers	3100
Basil, sweet	*Ocimum basilicum* L.	Leaves & stems	1500
Bene	*Sesamum orientale* L.	Flower tops & leaves	2200
Betony	*Lycopus virginicus* L.	Leaves	2750
Black walnut, bark*	*Juglans nigra*	Bark	2500

* Obtained through the courtesy of S. B. Penick & Company, New York, N. Y., and listed according to the names and spelling in their Price List and Manual of Botanical Crude Drugs, December 1, 1937, or on the package received.

† Obtained through the courtesy of Montague Free, Brooklyn Botanic Garden, Brooklyn, N. Y.

‡ A number of plant products including anise seed, black pepper, cloves, *Cocculus indicus*, larkspur, and wormwood appearing in this table are listed as possessing insecticidal properties to certain species of insects other than *Culex quinquefasciatus*, by N. E. McIndoo and A. F. Sievers in "Plants tested for or reported as possessing insecticidal properties," U. S. Dept. Agric. Dept. Bull. 1201. 61 pp. 1924.

** Obtained from Peter Henderson & Co., New York, N. Y.

†† Obtained from W. Atlee Burpee Co., Philadelphia, Pa.

TABLE I (*Continued*)

Common or trade name	Scientific name	Plant part	LD50 p.p.m.
Caraway seed, U.S.P.*	*Carum carvi*	Seeds	1000
Cayenne pepper		Fruit	1050
Chamomile, Hungarian, N.F.*	*Matricaria chamomilla*	Whole plant	2500
Cinnamon		Bark	2400
Cocculus indicus, N.F.*	*Anamirta cocculus*	Berries	1750
Coriander seed, Morocco N.F.*	*Coriandrum sativum*	Seeds	2300
Dandelion leaves*		Leaves	4000
Gerbera	*Gerbera jamesoni* Bolus	Stems	3300
Ginger root, Jamaica, U.S.P., select*	*Zingiber officinale*	Root	1600
Gourd (Dish-cloth)	*Luffa* sp.	Seeds	1225
Gourd, Turks' Turban	*Cucurbita maxima* Duchesne	Seeds	1500
Hyssop	*Hyssopus officinalis* L.	Flowers & stems	4000
Hyssop herb*	*Hyssopus officinalis*	Whole plant	3400
Kamala, genuine, N.F.*	*Mallotus philippinensis*	Hairs of capsules	2500
Koelreuteria†	*Koelreuteria apiculata* Rehd. & Wilson	Seeds	1400
Milkweed	*Asclepias syriaca* L.	Leaves	3600
Mugwort	*Artemisia vulgaris* L.	Tops & seeds	3500
Mullein flowers*	*Verbascum phlomoides* or *V. thapsiforme*	Flowers	4000
Muskmelon††		Seeds	1100
Mustard seed, yellow, English*	*Sinapis alba*	Seeds	1000
Nasturtium	*Tropaeolum majus* L.	Leaves & stems	4000
Peach leaves*		Leaves	2350
Plantago seeds*		Seeds	2700
Quince seed, Madagascar*		Seeds	1500
Sage	*Salvia officinalis* L.	Leaves & stems	4000
Senna pods*	*Cassia acutifolia* & *C. angustifolia*	Pods	2900
Senna, tinnevelly, small, U.S.P.*	*Cassia angustifolia*	Leaves	2900
Serpentaria root, U.S.P.*	*Aristolochia serpentaria* & *A. reticulata*	Root	2800
Sesame seed*		Seeds	1000
Stavesacre seed, recleaned*	*Delphinium staphisagria*	Seeds	1275
Trichosanthes	*Trichosanthes* sp.	Seeds	1500
Valerian root, U.S.P.*	*Valeriana officinalis*	Root	3800
Wafer ash bark of root*	*Ptelea trifoliata*	Bark of root	3600
Wild indigo root*	*Baptisia tinctoria*	Root	3500
Wild lettuce leaves*	*Lactuca virosa*	Leaves	3900
Wormwood	*Artemisia absinthium* L.	Leaves, stems & flower heads	2000
C. Plant products with LD50 values greater than 4000 p.p.m.			
Aconite root*	*Aconitum napellus*	Root	
Allspice		Dried unripe berries	
Aloes, socotrine, true*	*Aloe perryi*	Whole plant	
Areca nuts, N.F.*	*Areca catechu*	Fruit	
Arnica flowers, N.F.*	*Arnica montana*	Flowers	
Benzoin gum, Sumatra, U.S.P., select*	*Styrax benzoin*	Gum	
Butter and eggs	*Linaria vulgaris* Hill	Flowers & leaves	
Cascara sagrada bark, U.S.P., new*	*Rhamnus purschiana*	Bark	
Cascarilla bark, quills*	*Croton eluteria*	Bark	
Celandine	*Chelidonium majus* L.	Leaves & stems	

TABLE I (*Continued*)

Common or trade name	Scientific name	Plant part
Colocynth pulp, N.F.*	*Citrullus colocynthis*	Fruit
Dandelion root, N.F.*		Root
Dill	*Anethum graveolens* L.	Stems & leaves
Dittany herb, American*	*Cunila origanoides*	Whole plant
Dogwood, flowering	*Cornus florida* L.	Bark
Euphorbia herb	*Euphorbia* sp.	Leaves & stems
Fern, Maiden hair*	*Adiantum pedatum*	Whole plant
Fumatory herb*	*Fumaria officinalis*	Whole plant
Galega herb*	*Galega officinalis*	Whole plant
Geranium	*Pelargonium zonale* Willd.	Leaves
Henna leaves, Egyptian, true*	*Lawsonia inermis*	Leaves
Hops, N.F.*	*Humulus lupulus*	Whole plant
Horse radish root*	*Roripa armoracia*	Root
Iceland moss*	*Cetraria islandica*	Whole plant
Ipecac root, U.S.P.*	*Cephaelis acuminata*	Root
Jequirity seed*	*Abrus precatorius*	Seeds
Kousso flowers*	*Hagenia abyssinica*	Flowers
Laurel leaves, select*	*Laurus nobilis*	Leaves
Lavender spica	*Lavandula spica* L.	Flowers
Lavender, true	*Lavandula vera* DC	Leaves, stems & flowers
Lemon geranium	*Pelargonium crispum* L'Her.	Leaves
Licorice root, U.S.P. (Spanish)*	*Glycyrrhiza glabra typica*	Root
Lily of valley leaves*		Leaves
Lobelia herb*	*Lobelia inflata*	Whole plant
Logwood chips*		Wood
Lovage	*Levisticum officinale* Koch	Leaves & stems
Marigold, French (Harmony)	*Tagetes patula* L.	Flowers
Marigold, Yellow Pigmy, Dwarf Double French	*Tagetes patula* L.	Flowers
Milkweed	*Asclepias syriaca* L.	Stems
Mimulus	*Mimulus* sp.	Leaves & stems
Mullein leaves*	*Verbascum thapsus*	Leaves
Myrrh gum, U.S.P.*	*Commiphora myrrha* & species	Gum
Old man (see also southernwood herb)	*Artemisia abrotanum* L.	Leaves, stems & flowers
Olibanum tears, select, white*		Whole plant
Paraguay tea*	*Ilex paraguayensis*	Leaves
Parsley	*Petroselinum hortense* Hoffm.	Leaves & stems
Peppermint	*Mentha piperita* L.	Leaves, stems & spikes
Shepherd's purse*	*Capsella bursa pastoris*	Whole plant
Southernwood herb*	*Artemisia abrotanum*	Whole plant
Spearmint	*Mentha spicata* L.	Leaves, stems & spikes
Sumach leaves*	*Rhus glabra*	Leaves
Sweet fern	*Myrica asplenifolia* L.	Leaves & stems
Sweet woodruff	*Asperula odorata* L.	Leaves & stems
Thyme leaves, N.F.*	*Thymus vulgaris*	Leaves
Tragacanth aleppo, extra select*		Gum
Wild carrot seed*		Seeds
Wild cherry bark, U.S.P., Virgin green*	*Prunus serotina*	Bark
Witch hazel bark*	*Hamamelis virginiana*	Bark

vomica L. (24, p. 703), balsam of Peru, and rotenone in acetone solutions were tested on mosquito larvae. The following LD50 values were obtained

from these products: balsam of Peru 2000 p.p.m., ephedrine 2950 p.p.m., loganin 2375 p.p.m., and rotenone 0.06 p.p.m.

The toxicity of piperine, the toxic principle of black pepper, to houseflies (*Musca domestica* L.) has been reported by Harvill and others (13) in a previous publication. Piperine was found by them to be more toxic than pyrethrum to houseflies.

TOXICITY OF EXTRACTS TO APHIDS AND MEXICAN BEAN BEETLE

Acetone extracts of black pepper, prickly ash bark, and watermelon seeds were tested at a concentration of approximately 4000 p.p.m. on *Aphis rumicis* but gave unsatisfactory kills ranging from 38 to 53 per cent.

Tests made with Mexican bean beetle adults and larvae using 1.2 per cent of an acetone extract of black pepper emulsified with Penetrol (0.5 per cent) in water showed no appreciable kill. Acetone extracts of cubeb berries applied in a similar manner also were negative. When black pepper was dusted on bean plants and the beetles placed on the foliage, the kill obtained was 94 per cent. The number of insects used in individual tests was 25. Tests were run in duplicate. Counts of living and dead beetles were made 24 hours after treatment.

DISCUSSION

Plants known to contain poisons to humans were avoided, with few exceptions, in this study as it was hoped to find toxins specific to insects.

It is interesting to note that 10 of the 11 plant products giving the highest kill to mosquito larvae in this study were obtained from the flowers or fruiting parts of the plants. The 11 species of plants from which these products were obtained are distributed in 8 families. The highest kills were obtained with *Piper nigrum* and *Piper cubeba* both of which belong to the Piperaceae.

Plants that contain products that are toxic to mosquito larvae are widely distributed throughout the world. This suggests that should serious shortages of standard larvacides occur, local products may possibly be substituted.

The failure of both water and acetone extracts of sesame to be toxic except at relatively high concentrations is probably due to the fact that sesame has activating properties but is relatively non-toxic when used alone.

It is realized that both water and acetone methods of extractions have limitations and other methods must be employed also if an exhaustive search is to be made for insecticidal plants. Notwithstanding this, acetone extraction is useful in such a search as indicated by the fact that black pepper rates high in toxicity by this method. There is also the possibility that the use of the Soxhlet extractor for water extractions may be too

drastic a method for obtaining the insecticidal constituents. Both McIndoo (18) and Roark (21) have pointed out the advisability of using several solvents and the necessity of testing extracts on several species of insects in making a thorough search for plant insecticides.

SUMMARY

Plant products, upward to 125 species and varieties of plants, and three isolated toxins derived from plants, were tested for insecticidal properties. Of this number, acetone extracts of 11 species and varieties gave median lethal dose values (LD50s) between 24 p.p.m. and 520 p.p.m. to mosquito larvae (*Culex quinquefasciatus* Say). Water extracts of none of these products gave kills within this dose range.

The median lethal dose expressed in p.p.m. for mosquito larvae of acetone extracts of the 11 most promising insecticides tested, as compared with an LD50 of 0.06 for the isolated toxin rotenone, follow: cubeb berries 24, black pepper 29, *Koelreuteria paniculata* Lamx. seeds and leaves 245, elder flowers 300, jalap root 380, *Papaver* flowers and stems 440, and seeds of the following: Golden Summer Crookneck squash 450, Connecticut Straightneck squash 460, anise 470, Henderson cucumber 520, and honey dew melon 520.

Acetone extracts of black pepper, prickly ash bark, and watermelon seeds at concentrations of approximately 4000 p.p.m. failed to give satisfactory kills of *Aphis rumicis* L.

Negative results were also obtained with an acetone extract of black pepper (1.2 per cent) emulsified with Penetrol (0.5 per cent) in water when tested as a stomach poison on Mexican bean beetle (*Epilachna varivestis* Muls.).

Satisfactory kills were obtained with this insect when bean plants were dusted with black pepper and adults allowed to feed on the foliage under experimental conditions in the greenhouse.

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HISTOLOGICAL EFFECTS OF PIPERINE ON THE CENTRAL NERVOUS SYSTEM OF THE HOUSEFLY

ALBERT HARTZELL AND MURIEL STRONG

The histological effects of pyrethrum and isobutyl undecylene amide, an activator of pyrethrum, on the central nervous system and muscles of the housefly (*Musca domestica* L.) have been described by Hartzell and Scudder (2). Pyrethrum was demonstrated to have widespread clumping effect on the chromatin of the nuclei while isobutyl undecylene amide caused a chromatolysis or dissolution of the chromatin. A combination of these two agents showed a histological picture that was a summation of the effects of both. The interaction of these two types of nuclear destruction was believed to be the true basis of "activation."

The destructive action of pyrethrum on the central nervous system of insects has been reported also by Wigglesworth (6). Bugs of the species *Rhodnius prolixus* that had been paralyzed for ten days by the application of pyrethrum in liquid paraffin to the antennae showed degeneration in the fused abdominal ganglia when the tissue was fixed in Carnoy's solution and stained with haematoxylin. He demonstrated that there was no increase in evaporation from insects paralyzed with pyrethrum until after death, and that the spiracles are kept closed and still react to carbon dioxide. Desiccation, he concluded, cannot be the main cause of death after pyrethrum poisoning.

Recently Harvill and others (3) have reported that piperine, the alkaloid found in the dried fruit of black pepper (*Piper nigrum* L.), is more toxic to houseflies than pyrethrum, but except at high concentrations lacks the property of quick knockdown characteristic of pyrethrum.

The present investigation was made to determine whether piperine is a neurotoxic poison to houseflies which might be detected by staining the fiber tracts with silver albumose and gold chloride following Bodian's technique (1).

MATERIALS AND METHODS

Adult houseflies, five days old, of both sexes were sprayed under regular Peet-Grady test conditions (5) with a relatively high concentration (0.5 per cent) of piperine in "Deo-base" containing 10 per cent absolute alcohol. At this concentration of piperine a knockdown of 89 per cent was obtained in 10 minutes. Moribund individuals which were capable of slight movements on probing were collected at intervals of 10, 60, 120, and 180 minutes after spraying. At 180 minutes both the flies which had recovered sufficiently to be able to walk and the dead individuals were collected. Twenty

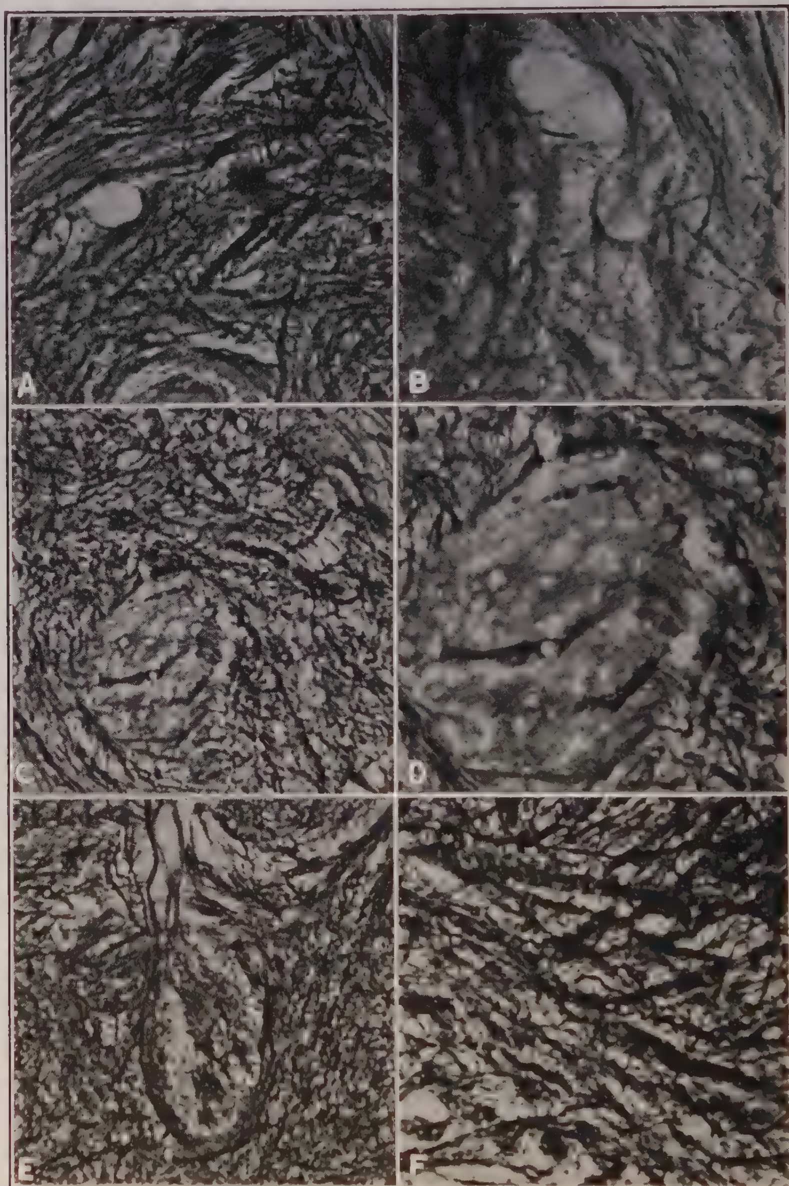

FIGURE 1. Vertical sections of the brain in the region of the corpus centrale and the fiber tracts lateral of it, of the housefly, fixed in Dietrich's solution and stained with the silver albumose and gold chloride method of Bodian. Figures on left, ×510; figures on right, ×1020. A and B, check. C and D, showing the effect of piperine 10 minutes after spraying. The nerve fibers show evidence of dissolution but no marked degeneration. E and F, showing the effect of piperine two hours after spraying. Note vacuolization of tissue and degeneration of nerve fibers.

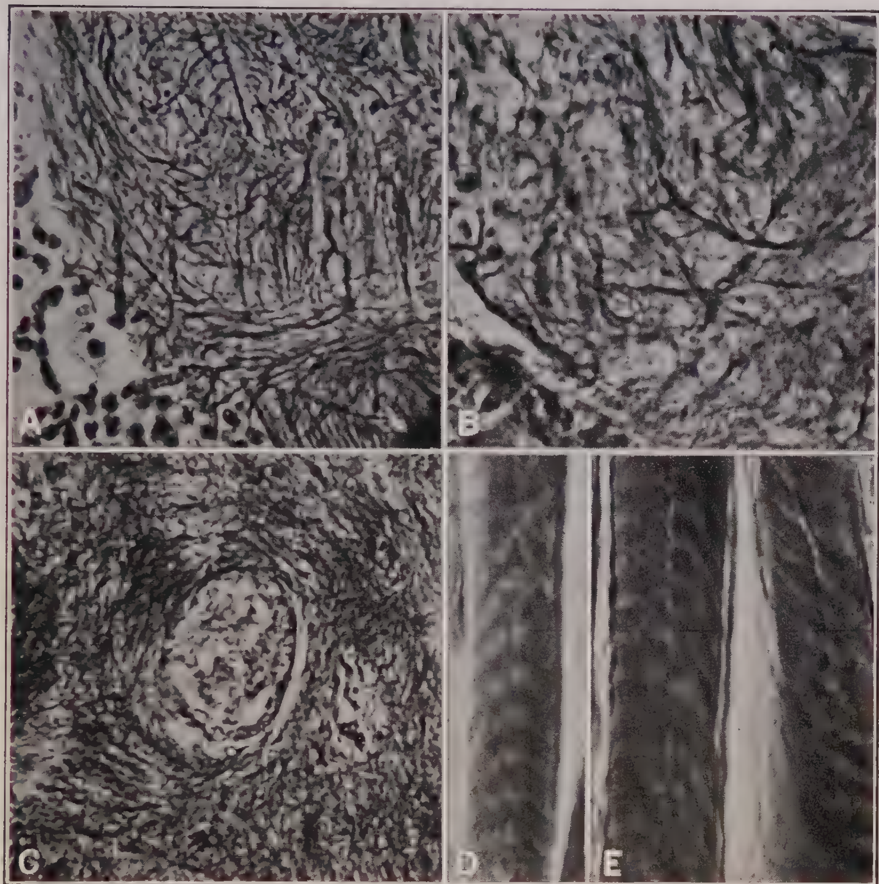

FIGURE 2. A. Vertical sections of the brain in the region of the corpus centrale and the fiber tracts lateral of it, of the housefly, fixed in Dietrich's solution and stained with the silver albumose and gold chloride method of Bodian. Recovering at 20 hours after spraying with piperine. Note absence of degeneration and that the nerve axons appear normal, ×580. B, as in the preceding figure but at higher magnification, ×1160. C, showing the effects of piperine in a dead individual three hours after spraying with piperine, ×580. Note the destruction of the fiber tracts as indicated by darker staining. Muscles of the head of housefly fixed in Dietrich's solution and stained with iron haematoxylin and erythrosin, ×1160. D, check. E, showing the effect of piperine 10 minutes after spraying. Note that the muscles are in a state of tension and that Krause's membrane is accentuated.

hours after spraying, the flies which had fully recovered also were collected. Immediately after collecting the flies, the tissues were killed and fixed in Dietrich's solution and the abdomens were punctured by means of a needle to allow complete permeation. Dehydration was accomplished by the *n*-butyl alcohol technique. The tissue was imbedded in (60° to 62° C. m.p.) paraffin. Sections of whole insects including head and thorax, but with the wings removed, were cut at 10μ and stained by the Bodian technique (1) for the study of nerve fibers and by the iron haematoxylin and erythrosin method (4, p. 71) for the study of muscles. Controls were subjected to the same methods for a comparison of the normal histology.

RESULTS

The foremost effect of piperine on the brain of the housefly in moribund individuals was the destruction of the fiber tracts, especially in the region of the corpus centrale, and vacuolization of the nerve tissue (Fig. 1, compare A and B with E and F). Specimens collected ten minutes after spraying (Fig. 1 C and D) showed evidence of the dissolution of the fibers but no marked degeneration of the nerve tissue. In dead individuals collected three hours after spraying (Fig. 2 C) there was drastic disintegration of nerve tissue as indicated by differential staining into light and dark areas. The nerve axons of individuals which had recovered sufficiently to fly (collected 20 hrs. after spraying) showed an almost complete absence of degeneration effect (Fig. 2 A and B).

The head muscles of houseflies that were sprayed with piperine (0.5 per cent) showed evidence of tetanus and Krause's membrane appeared to be enlarged so that it stood out very prominently (Fig. 2, compare D and E).

DISCUSSION

Piperine like pyrethrum exhibits its foremost effect in the destruction of the fiber tracts of the brain of the housefly. The widespread clumping effect of the chromatin of the nuclei which is characteristic for pyrethrum (2) was not observed with piperine. This would suggest that the effect of piperine on the housefly is much less general than with pyrethrum.

The action of piperine on the muscles of the housefly also appears to be different from pyrethrum. With pyrethrum there is fenestration of the cytoplasm, clumping of the chromatin of the nuclei, and loss of striation of the muscles while with piperine the foremost effect is the enlargement and accentuation of what appears to be Krause's membrane. It should be borne in mind that the concentration of piperine used in this study was considerably higher than would be normally used in fly sprays.

SUMMARY

Piperine was found to show distinct and characteristic effects on the

central nervous system and associated muscles of the housefly in a preliminary histological study.

The foremost effect was the destruction of the fiber tracts and vacuolization of the nerve tissue of the brain, but the widespread clumping effect of the chromatin of the nuclei characteristic for pyrethrum was not observed with piperine.

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SOME SEEDS SHOWING SPECIAL DORMANCY

Lela V. Barton

INTRODUCTION

Certain seeds have been found to possess a special kind of dormancy which has been called epicotyl dormancy. Reference in these cases is to the failure to produce shoots above ground even after the roots have appeared and have become established in the soil. It has been found necessary to expose the germinated seed, with the root system beginning to develop, to a temperature of 1° to 10° C. for a while in order to after-ripen the epicotyl or the bud which forms it. Seeds of the tree peony, *Paeonia suffruticosa* Andr. (2), some of the lilies, *Lilium auratum* Lindl., *L. canadense* L., *L. japonicum* Thunb., and others (3), and certain of the Viburnums such as *Viburnum acerifolium* L. and *V. prunifolium* L. (6) are known to exhibit this type of dormancy.

Convallaria majalis L. and *Smilacina racemosa* (L.) Desf. seeds show epicotyl dormancy of a rather specialized type in that the period at low temperature must be given, not merely after root production, but after the shoot has started to develop and has broken through the cotyledonary sheath (4). Exposure to low temperatures at earlier developmental stages was without effect in breaking epicotyl dormancy. Furthermore, low temperature pretreatment of the moist seeds increased the root production from seeds of *Convallaria* and was essential to root formation in *Smilacina* when plantings were made in soil in the greenhouse. These facts pointed to a double dormancy. An initial treatment of the seeds at low temperature was required to after-ripen the root, followed by a period at high temperature during which the root system grew and the shoot developed until it had broken through the cotyledonary sheath. A second period at low temperature was then necessary to after-ripen the epicotyl, followed by a second period at high temperature during which the first green leaves appeared above soil.

Thus different seeds are seen to exhibit varying types and degrees of dormancy both of the root and of the epicotyl.

The present report includes studies made on *Asarum canadense* L., *Sanguinaria canadensis* L., *Polygonatum commutatum* (R. & S.) Dietr., *Trillium grandiflorum* (Michx.) Salisb., *Trillium erectum* L., and *Caulophyllum thalictroides* (L.) Michx. The seeds of all of these forms have epicotyl dormancy requiring a period at low temperature after root formation to permit development of the green shoots. Some of them also need pretreatment at low temperature before roots will be produced, thus showing

the double dormancy described above. Different degrees of dormancy are illustrated by the seeds of these various forms.

RESULTS AND DISCUSSION

Asarum canadense. Seeds of the wild ginger have the simple type of epicotyl dormancy shown by those of tree peony (2).

Seeds were collected at Yonkers, New York on June 3, 1942. They were cleaned and while still moist were placed in moist granulated peat moss at constant temperatures of 5°, 15°, 20°, 25°, and 30° C. as well as at daily alternating temperatures of 10° to 20° C., 10° to 30° C., 15° to 30° C., and 20° to 30° C. The best root production, 77 per cent, obtained was at a daily alternating temperature of 10° to 20° C. Most of these seeds had germinated by the end of two months. Ten degrees to 30° C. and 15° to 30° C. were less effective and 20° to 30° C. inhibited germination. Fifty-two and 41 per cent of the seeds germinated at constant temperatures of 15° and 20° C., respectively, and none germinated at constant 25° or 30° C. Pretreatment at 5° C. for one to six months failed to increase subsequent germination at 20° C. or 10° to 20° C.

It appeared that seedling production from seeds of *Asarum canadense* was a simple process but when germinated seeds with the roots growing normally were planted in soil in the greenhouse only occasional green shoots appeared above the soil and these grew slowly. Consequently germinated seeds planted in soil in pots were given further treatment for one, two, three, and five months at 5° or 10° C. The soil was kept moist throughout these periods. Here the epicotyl after-ripened so that green leaf production followed immediately upon transfer from the low temperature to the greenhouse. After three months at 5° C., for example, 80 to 95 per cent of the seedlings produced green leaves within two weeks after transfer to the greenhouse. Five months at 5° C. was too long since the seedlings came above soil in that time at the low temperature. Ten degrees C. was also effective for after-ripening the epicotyl. Periods of one, two, or three months could be used for after-ripening at this temperature. When one month only was used, the green leaf production was delayed upon transfer to the greenhouse so that two months were required for a complete seedling stand. When two or three months' after-ripening periods were used, only two weeks in the greenhouse were required for complete green leaf production.

At the same time that seeds were mixed with granulated peat moss other samples of the same lot were planted in four-inch pots in soil. Some of these pots were placed in a room at 5° C. and others were taken to the greenhouse immediately after planting. Again it was demonstrated that the initial period at low temperature was of no advantage in root production but low temperature after the root appeared was necessary to over-

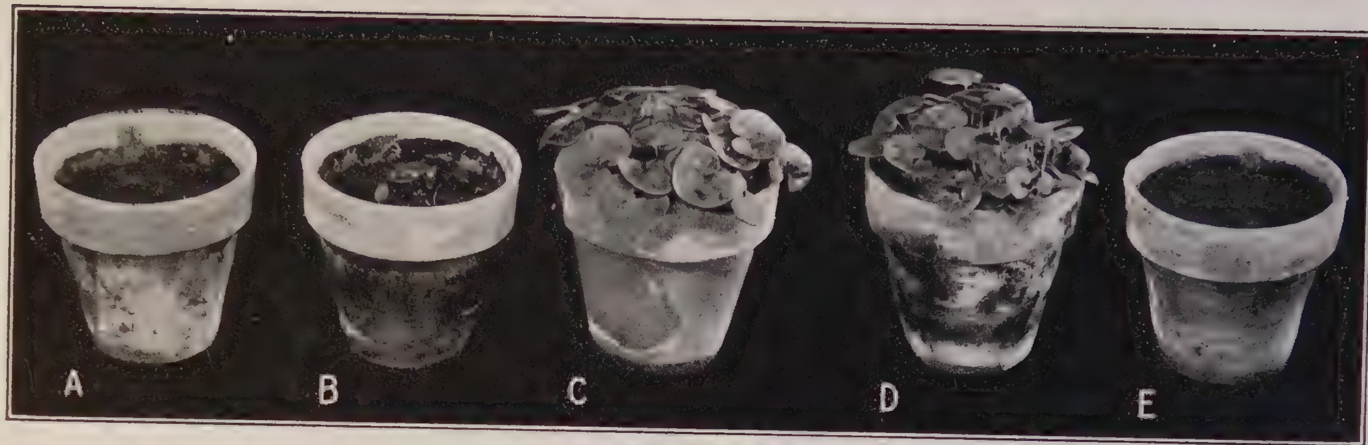

FIGURE 1. *Asarum canadense*. One hundred seeds each planted in June 1942 and photographed in March 1943. A. Greenhouse control; B. greenhouse three months +5° C. one month, then replaced in the greenhouse October 9, 1942; C. greenhouse three months +5° C. three months, then replaced in the greenhouse December 5, 1942; D. 5° C. one month+greenhouse three months+5° C. three months, then replaced in the greenhouse January 5, 1943; E. 5° C. three months+greenhouse one month + 5° C. three months, then replaced in the greenhouse January 5, 1943.

come shoot dormancy. This may be seen in Figure 1. All of these seeds, 100 in each pot, were planted on June 5, 1942, and the photographs were made on March 16, 1943. Three very small green leaves had appeared in the greenhouse control at this time (Fig. 1 A). An examination revealed that this pot had well developed seedlings underground on the preceding November 5, but green shoot development was prevented by epicotyl dormancy. Three months in the greenhouse were sufficient to bring about germination of the seeds to form roots. Shorter periods were less effective. Certain pots were removed from the greenhouse three months after planting and were placed in a room at 5° C. for one or three months after which they were replaced in the greenhouse. Green leaf production after such treatment is shown in Figure 1 B and C. It will be seen that one month at 5° C. was insufficient to after-ripen the epicotyl completely since few and slow-growing green leaves were produced (Fig. 1 B). Satisfactory after-ripening occurred in three months at 5° C. as evidenced by complete green leaf production two weeks after transfer to the greenhouse. Furthermore, after such treatment the seedlings grew rapidly and normally (Fig. 1 C). This pot was transferred to the greenhouse on December 5, 1942. The pot in Figure 1 B was transferred to the greenhouse on October 9, 1942, but seedling growth was poor due to incomplete after-ripening.

In Figure 1 D and E is shown the influence on root production and hence subsequent shoot production of an initial treatment of the seeds at 5° C. for one and three months followed by three and one months, respectively, in the greenhouse before exposure to 5° C. for three months to break epicotyl dormancy. Treatment of the seeds at 5° C. was without effect. It neither decreased the time required in the greenhouse for root formation nor increased the subsequent green shoot production over that shown in Figure 1 C.

Sanguinaria canadensis. Seed behavior in this form differs from that described above for *Asarum* in that pretreatment of the seeds in a moist medium at 5° C. for three months resulted in increased root production over that obtained from untreated seeds. That low temperature pretreatment of the seeds was not absolutely essential for germination to form roots, however, was shown by the behavior of untreated seeds in moist granulated peat moss at a constant temperature of 20° C. where a final germination percentage of 51 was obtained. Thirty-nine per cent of the untreated seeds produced roots at 20° C. in four months and germination thereafter was sporadic so that 51 per cent resulted after a year. Also, flat plantings made out-of-doors in June 1936 and wintered in a board-covered frame with a temperature range from 3° to 5° C. yielded 33 per cent green plants in June 1937. The roots of these plants had emerged from the seeds by the end of the summer after they were planted and the winter cold permitted epicotyl after-ripening so that green shoots were produced the following spring. Because some roots were produced from seeds without

low temperature pretreatment but an increased number (up to 90 per cent) resulted if the seeds were pretreated at 5° C. for three months, the root may be said to be partially dormant.

Sanguinaria seeds also differ from *Asarum* seeds in the length of time, six months instead of three, required to grow the young seedlings with the root already formed to the stage where a second period of three months at 5° C. will effectively after-ripen the epicotyl.

Burunjik (5) reported a complete lack of germination of the seeds of *Sanguinaria canadensis* when the coats were untouched, but said that the coat was not the only limitation since several months were still necessary for after-ripening. Coat treatment consisted in removal of the coats or dipping for three minutes in boiling water. She obtained 60 per cent germination in 29 months when the seeds were planted in soil. Sulphuric acid used on the seed coats in the present study was ineffective in hastening germination. Germination behavior indicated that temperature was the most important factor.

Polygonatum commutatum. These seeds were similar in germination response to those of *Sanguinaria canadensis*, described above, and *Convallaria majalis* (4). If soil plantings in the greenhouse were used, roots were produced only after low temperature pretreatment but up to 42 per cent of the untreated seeds germinated to form roots in moist granulated peat moss at 25° C. in two months with only occasional germinations after that time. However, the percentage at 25° C. was increased to 90 if the seeds were kept moist at 5° C. for four months prior to transfer to 25° C.

Plantings made out-of-doors produced seedlings above ground only after two winters. The first winter after-ripened the root, the following summer permitted the root system to become established, the second winter after-ripened the epicotyl, and green leaves were produced the second spring. No green leaves ever developed without exposure of the germinated seed to low temperature.

These seeds represent then still another form with partial root dormancy and complete epicotyl dormancy.

Trillium grandiflorum. The results of experiments with seeds of *Trillium grandiflorum* demonstrated complete double dormancy or dormancy of both root and shoot, necessitating two separate periods at low temperature to after-ripen.

Clean seeds of the 1940 crop of this species, collected at Yonkers, New York were planted in pots on July 25, 1940. One hundred seeds were planted for each treatment. For the most part single pots were used but three control lots were kept in the greenhouse for the entire period of the test. The greenhouse temperature during periods when it could be controlled was 21° C. None of the seeds in these pots were able to produce green seedlings within a period of 14 months when the experiment was terminated.

Other pots were placed at 5° C. immediately after planting where they

were kept for one, two, or three months, after which they were transferred to the greenhouse. One of these pots which had been at 5° C. for three months was kept in the greenhouse for 11 months. Three green seedlings appeared in this pot after eight months but no more green shoots were produced. Furthermore, these seedlings grew very slowly.

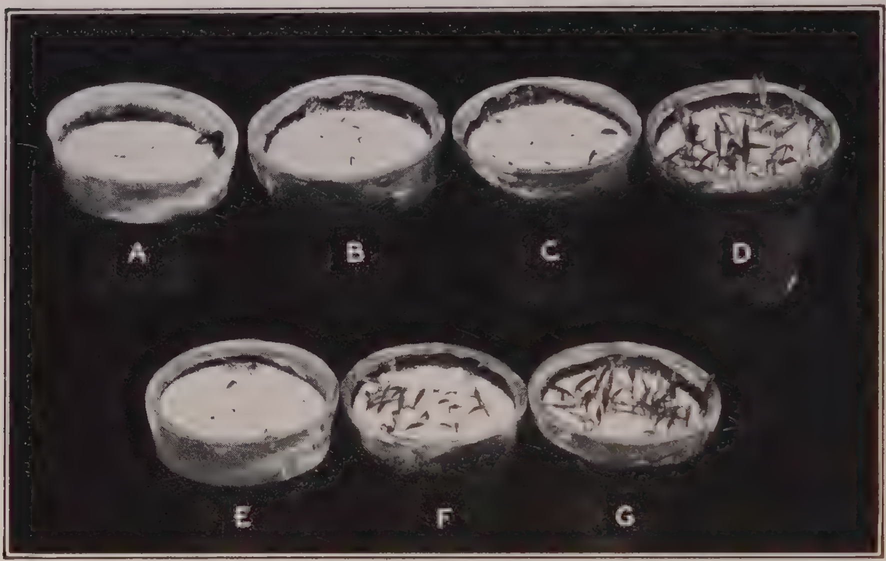

FIGURE 2. *Trillium grandiflorum*, 1940 crop. One hundred seeds planted in each pot. Top row: 5° C. three months+greenhouse three months+5° C. for (A) 0, (B) 1, (C) 2, and (D) 4 months, then replaced in the greenhouse. Bottom row: Initial treatment at 5° C. for (E) one month, (F) two months, and (G) three months followed by greenhouse three months+5° C. three months, then replaced in the greenhouse.

Other pots kept for an initial period of three months at 5° C. were transferred to the greenhouse for three months. Although no green seedlings had appeared at this time, many of the seeds had germinated and had formed good root systems. These pots were then replaced at 5° C. for one, two, three, or four months after which a second transfer to the greenhouse was made. The result of such treatment is shown in Figure 2. Here the effect of an initial period of three months at 5° C. followed by three months in the greenhouse plus a second period of zero, one, two, or four months at 5° C. on green shoot production after a final transfer to the greenhouse is demonstrated. That the initial period of three months at 5° C. was adequate for after-ripening the seed so that the roots would grow and develop is clearly shown (Fig. 2 D). It is also seen that a second period at 5° C. was necessary for after-ripening the epicotyl and that one or two months was insufficient time for this purpose, as indicated by the few green shoots

produced after such treatment (Fig. 2 A, B, and C). A second period of four months at 5° C., however, was effective in bringing about green leaf production (Fig. 2 D).

In Figure 2 E, F, and G are shown the results from an initial 5° C. treatment of one, two, or three months. The requirements for epicotyl after-ripening and green shoot production, i.e., three months in the greenhouse plus three months at 5° C. following the initial treatment, were met for all of these seeds, but the initial treatment was varied to determine effect on root production. The necessity for after-ripening the seed for root production is clearly demonstrated. One month was ineffective and a two-month period was not long enough to cause good germination although some root production was obtained. Three months was an adequate time to allow. A second period of three months resulted in good green shoot production (Fig. 2 G) although four months was not injurious (Fig. 2 D).

TABLE I

TRILLIUM GRANDIFLORUM 1941 CROP. ROOT AND SHOOT PRODUCTION AFTER VARIOUS TEMPERATURE TREATMENTS FROM DUPLICATE LOTS OF 100 SEEDS EACH PLANTED IN SOIL IN POTS. ALL PERCENTAGES BASED ON TOTAL NUMBER OF SEEDS PLANTED

Treatment					Per cent seedling production			
First period at low temperature		Months in greenhouse	Second period at low temperature		Underground		Above ground	
° C.	Months		° C.	Months	a	b	a	b
None	None	17	None	None	7	6	1	0
None	None	6	10	3	35	32	0	0
5	3	3	5	3	83	84	54	38
	3	3	5	5	90	86	80	80
10	3	3	10	3	86	78	63	46
	3	3	10	5	69	81	63	72

Some of the seeds of the 1940 crop were dried for two weeks and then planted. Although the response to temperature treatment was the same as for moist seeds, the germination percentages obtained were lower, indicating some harmful effect of drying.

Seeds were collected again in 1941 from the same source as the 1940 crop. More seeds were secured and more extensive tests were conducted. Duplicate pots of 100 seeds each were used for each test. The results are shown in Table I. In addition to 5° C., 10° C. was employed for after-ripening. The initial period at low temperature was extended up to nine months in some cases to see whether the second period at low temperature and the intervening period in the greenhouse could be eliminated. At the

termination of the experiment all of the pots were sieved so that the number of seedlings underground as well as the green ones above ground could be ascertained.

When the seeds were placed in the greenhouse and left there for 17 months without any further treatment, 7 and 6 seedlings grew below ground in the duplicate pots and one green shoot appeared above ground. When seeds planted and placed directly in the greenhouse were left there for six months and then transferred to 10° C. for three months after which they were replaced in the greenhouse, about 32 and 35 per cent root production resulted but no green shoots were obtained. This indicated the response of the root to after-ripening but failed to provide the second low temperature period necessary for normal green shoot production.

Other effects indicated in Table I are similar to those pictured in Figure 2. When the second period at 5° C. was extended to five months instead of three, a higher percentage of green shoots appeared above ground. This effect was independent of the root production as can be seen in the table. A total of 83 to 90 per cent root production took place in either case. This was to be expected since the initial or root after-ripening period was adequate and was the same in both cases. An extension of the initial period at low temperature to six or nine months was without effect on subsequent green leaf production. Furthermore, these longer periods were either without effect on root production or resulted in a slight increase in the number of roots formed. No harmful influence was noted. Ten degrees C. was found to be as good as 5° C. for both root and epicotyl after-ripening.

Some of the seeds of the 1941 crop were cleaned and dried in the laboratory for a month before they were planted. Again it was demonstrated that drying was harmful to *Trillium grandiflorum* seeds. Planting the seeds while still fresh is of importance in producing good stands of seedlings.

Germinated seeds with roots starting to develop in moist granulated peat moss at 20° C. after 5° C. pretreatment were planted in pots and placed at constant temperatures of 5°, 10°, 15°, 20°, and 30° C. and a daily alternating temperature of 15° to 30° C. Transferred to a 15° C. greenhouse after four months at these various temperatures, green leaf production percentages were 40, 85, 85, 71, 0, and 52, respectively. Thus 15° C. as well as 5° and 10° C. proved effective for after-ripening the epicotyl. The appearance in the 15° C. greenhouse of green leaves in pots which had been at 20° C. or 15° to 30° C. for four months was delayed several weeks, indicating that these temperatures were not low enough for after-ripening but not high enough to be harmful and the 15° C. temperature of the greenhouse permitted continuance of growth. The roots rotted at a constant temperature of 30° C.

Seeds of *Trillium* planted out-of-doors and kept in mulched or board-

covered frames over winter required two winters for green leaf production and again indicated the presence of double dormancy.

The after-ripening effects on root production are in agreement with those reported by Burunjik (5) who stated that with low temperature treatment, *Trillium grandiflorum* seeds germinated 100 per cent in four months. She did not give experimental details but it is presumed that her figures apply to root production only.

Trillium erectum. Seeds of *Trillium erectum* were collected at Jamaica, Vermont, September 2, 1940. Clean seeds which had dried on blotters in the laboratory for 15 days were planted in pots. One hundred seeds were used for each pot. The control lot which was kept for a year in the greenhouse failed to produce any seedlings below or above the soil. Other lots were placed in a room kept at 5° C. where they were allowed to remain for three, six, or nine months after which they were transferred to the greenhouse. Certain of these were kept in the greenhouse for the remainder of the tests where a year later the green shoot production was 0, 7, and 11 per cent after three, six, and nine months at 5° C., respectively. These seedlings were very small and grew slowly. Other pots which had received an initial period of three, six, or nine months at 5° C. were kept for one or two months in the greenhouse after which they were removed again to the 5° C. room for one or three months. A second transfer to the greenhouse at the end of these periods resulted, in some cases, in up to 50 per cent green shoot production. Several effects were noticed. In the first place, the initial period at 5° C. needed to be at least six months in order to after-ripen the roots so that they would grow in the greenhouse. Secondly, either one or two months in the greenhouse was sufficient to grow the roots when the preceding time at 5° C. was as long as nine months. In the third place, a second period at 5° C. of one or three months was adequate for after-ripening the epicotyl after the root system had been formed.

Burunjik (5) reported that *Trillium erectum* gave no germination after seven months when fresh seeds were used but with six months' dry storage the seeds gave 70 per cent in five months. She does not state the germination temperature used or whether the percentages apply to root or shoot production. The favorable effect of dry storage has not been noted for seeds described in the present study.

Caulophyllum thalictroides. Over a period of several years numerous tests have been run to determine the germination requirements of these seeds. There is no doubt that the epicotyls are dormant. Furthermore, the type of epicotyl dormancy resembles that reported for *Convallaria majalis* and *Smilacina racemosa* (4) in that a certain stage of development of the young embryo must be reached before exposure to low temperature to enable after-ripening to proceed. This stage is indicated in Figure 3 which shows the enlarged hypocotyl split and the shoot bud exposed. After the

protrusion of the root from the seed, development to this stage took place in about a month in soil in the greenhouse. If the seedlings were allowed to remain at greenhouse temperature, an occasional green shoot was produced but remained dwarfed and failed to develop normally even after two years.

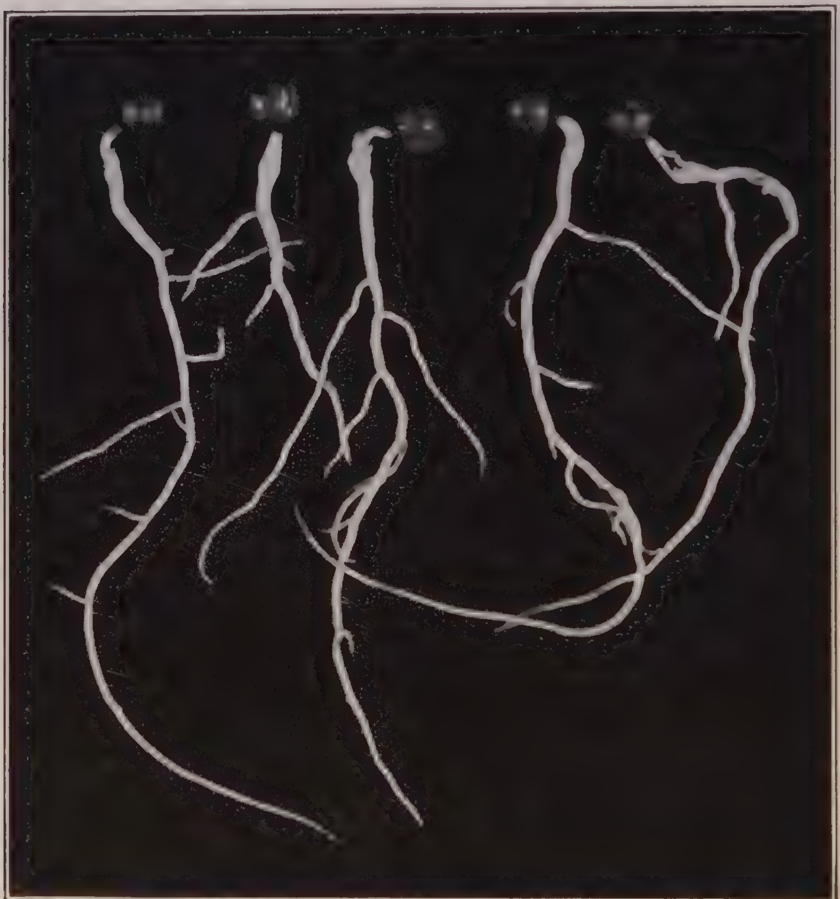

FIGURE 3. *Caulophyllum thalictroides.* Young seedlings planted in soil in the greenhouse, removed and photographed after one month.

The effects of various temperature treatments on epicotyl dormancy are shown in Table II. It should be kept in mind that seedlings with the root just beginning to develop were planted in soil in pots which were exposed to the various temperatures. The soil in these pots was kept moist throughout the tests. No seedlings appeared above ground in those pots kept in the greenhouse for six months although 66 per cent of them re-

mained alive and developed extensive root systems below the surface of the soil. Very young seedlings planted in soil and placed directly at low temperature failed to develop in five months at 5° C. to the stage necessary for after-ripening to proceed but 73 to 90 per cent continued to grow underground. At 10° C. more development took place so that in some of the

TABLE II

CAULOPHYLLUM THALICTROIDES. FURTHER DEVELOPMENT IN THE GREENHOUSE OF SEEDLINGS FROM GERMINATED SEEDS AFTER VARIOUS TEMPERATURE TREATMENTS. ALL PERCENTAGES BASED ON TOTAL NUMBER OF SEEDLINGS PLANTED

Time in greenhouse preceding low temp.	Low temperature		No. of seedlings planted	% Seedling production in greenhouse	
	° C.	Months		Underground	Above ground
6 mos.	None	None	95	66	0
None	5	1	14	86	0
		2	45	84	0
		3	42	90	0
		5	48	73	0
	10	1	11	73	0
		2	45	92	3
		3	41	87	24
		5	42	55	32
2 wks.	5	3	25	80	8
	10	3	25	68	60
1 mo.	5	2	25	92	0
		3	16	94	25
	10	2	25	56	36
		3	16	62	62
	15	3	38	44	31
6 wks.	5	3	20	*	15
	10	3	20	*	60
2 mos.	5	3	28	43	43
	10	2	140	37	37
		3	74	48	48
3 mos.	5	3	34	*	0
	10	3	34	*	0

* Not examined for seedlings underground.

seedlings after-ripening occurred after three to five months. However, a large percentage in this case also remained underground with no development of the green shoot. A period of two weeks in the greenhouse preceding

transfer to 5° or 10° C. permitted 8 per cent after-ripening of the epicotyls at 5° C. and 60 per cent at 10° C. No additional after-ripening was obtained at 10° C. even when the preceding period in the greenhouse to allow development of seedlings was extended to two months, but a beneficial effect of these longer periods in the greenhouse was evidenced when 5° C. was used as the after-ripening temperature. At least three months at 5° or 10° C. were required to break the epicotyl dormancy, so that green leaves were produced upon transfer to the greenhouse.

Although epicotyl dormancy behavior in the seeds of this species was determined by this study, root production presented a problem and is still not thoroughly understood. Clean seeds were placed at constant temperatures of 15°, 20°, 25°, and 30° C. and daily alternating temperatures of 10° to 20° C., 10° to 30° C., 15° to 30° C., and 20° to 30° C. The only germination obtained was at 15° to 30° C. and then only 11 per cent in 19 months.

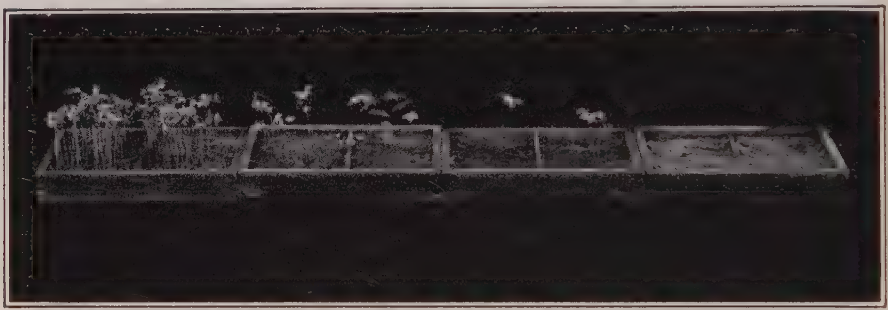

FIGURE 4. *Caulophyllum thalictroides*. Second-year growth of seedlings produced from seeds planted in a board-covered cold frame (left to right): September 22, October 22, November 22, and December 22, 1937. Duplicates of 100 seeds each planted in each flat. Photographed April 1941.

Various other exposures of intact and sulphuric acid-treated seeds to controlled temperatures have resulted in a maximum root production of 47 per cent. This was obtained from intact seeds left in moist granulated peat moss at 15° to 30° C. for three months then transferred to 10° C. for nine months and then back to 15° to 30° C. where the seedlings appeared within a month. Other instances where the seeds were placed first at 10° C., before the treatment described above, failed to give any better germination or throw any further light on the root-production requirements.

Fairly good green shoot production was obtained from outside plantings of clean seeds made in flats kept in board-covered cold frames giving temperatures of 3° to 5° C. over winter. Seeds thus planted on September 22, 1937, yielded green shoots in the spring of 1940 to the extent of 67 to 71 per

cent. No seedlings appeared above ground before that time and no additional seedlings appeared in the spring of 1941. A planting of the same lot of seeds on October 22, November 22, and December 22, 1937, yielded only 21, 4, and 0 per cent green seedlings in 1940, with no additional seedlings in 1941.

This effect of planting time may be due to the rapid deterioration of the seeds in dry storage in the laboratory. Figure 4 shows seedlings from these plantings as they appeared in 1941.

Difficulties in the germination of *Caulophyllum thalictroides* were also encountered by Adams (1), who planted seeds in September and obtained 30 per cent germination after 723 days. No germination was obtained in 549 days from seeds collected in September and planted the following March. On September 3, 1926 he examined 50 seeds which had been planted in 1924 and found that 12 had produced long branching roots, 3 were just sprouting, 28 were still hard and sound, and the remainder had decayed.

SUMMARY

A study of the germination behavior of seeds of *Asarum canadense*, *Sanguinaria canadensis*, *Polygonatum commutatum*, *Trillium grandiflorum*, *Trillium erectum*, and *Caulophyllum thalictroides* has shown that all of them possess epicotyl dormancy and require exposure to temperatures of 5° to 10° C. after the root has been formed to overcome this dormancy.

Seeds of *Asarum* exhibit no dormancy of the root, those of *Sanguinaria* and *Polygonatum* show partial dormancy of the root, while those of *Trillium grandiflorum*, *T. erectum*, and *Caulophyllum* have dormant roots as well as dormant epicotyls. Dormant roots may be induced to develop by moist low temperature pretreatment of the seeds. Thus *Trillium grandiflorum*, *T. erectum*, and *Caulophyllum* seeds have a double dormancy overcome by two separate cold periods, one before the appearance of the root and the other after germination to form a root has already taken place.

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XYLENOXY GROWTH SUBSTANCES

P. W. ZIMMERMAN, A. E. HITCHCOCK, AND E. K. HARVILL

The substitution of nitro, amino, and halogen radicals in the ring of phenoxy derivatives of the lower fatty acids has recently been shown to be an effective means for increasing the physiological activity of the molecule (3, 4, 5). The kind and degree of activity varies with the radical and its position in the ring. The present report is concerned with phenoxy acids having two methyl groups substituted in various positions in the ring. These compounds are called also xylenoxy acids.

A list of xylenoxy acids synthesized in the Boyce Thompson Institute laboratories is shown in Table I. They have been tested for physiological activity on *Lycopersicon esculentum* Mill. (tomato), *Nicandra physalodes* (L.) Pers., and *Cleome spinosa* L. Some of them have been tested for their capacity to induce root growth and parthenocarpy. The usual method of application described for substituted phenoxy acids has been followed (3, 4). The substances were effective when applied as lanolin preparations, water solutions, hot-plate aerosols, or vapors. Aerosol and vapor induced responses were similar. Root-inducing activity of the acids was determined with tomato leaf cuttings. The basal part of the excised leaf was placed in water solutions of the chemicals for 24 hours and then grown in water for six to eight days according to the methods previously described (1).

The activity of the xylenoxy acids for inducing parthenocarpic fruit of tomatoes was determined by spraying water solutions on the open flowers. Lanolin preparations served for determining the activity for cell elongation or modification (formative influence) of leaves. One application applied to one side of the stem and the upper side of the adjacent petiole was sufficient to determine within a few hours the capacity to induce curvatures through cell elongation and later formative effects as the plants grew. The latter response usually became evident on the new leaves three or more days after the plant was treated. The modified leaves differed from normal in size, shape, pattern, venation, and texture.

The comparative activity of xylenoxy acids for their power to induce cell elongation (causing curvatures from unilateral application) and for their formative influence is shown in Table I. The acids, salts, and esters are approximately equal in activity.

The physiological activity appears to be related to the kind, number, and position of the substituted groups in the benzene ring. In general, halogen substitutions appear to bring about greater activity than methyl, amino, or nitro groups. Regardless of the substitutions, however, the posi-

TABLE I

MOLECULAR CONFIGURATION AND PHYSIOLOGICAL ACTIVITY OF XYLENOXY COMPOUNDS

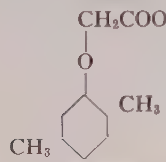
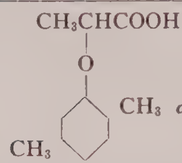
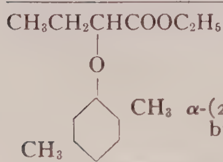
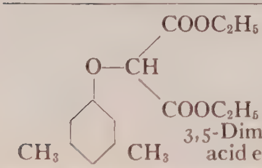
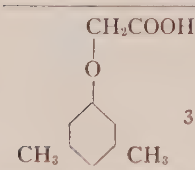
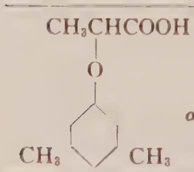
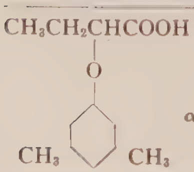
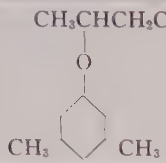
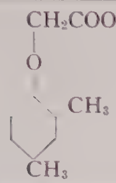
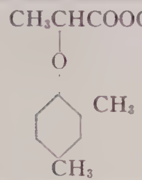
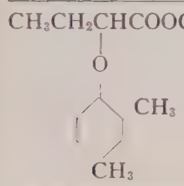
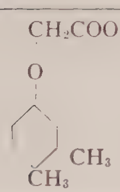
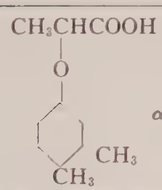
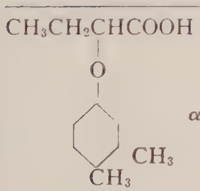
Xylenoxy acids and esters	Melting point, ° C.	Cell elongation and epinasty of tomato leaves. Threshold conc. mg./g. of lanolin	Modification of leaves. Threshold conc. mg./g. of lanolin
 2,5-Dimethylphenoxyacetic acid	115–115.5	Active 5	Inactive at 20 mg./g.
 α-(2,5-Dimethylphenoxy)-propionic acid	102–103	Active 0.5	Inactive at 20 mg./g.
 α-(2,5-Dimethylphenoxy)-*n*-butyric acid ethyl ester		Active 5	Inactive at 50 mg./g.
 3,5-Dimethylphenoxymalonic acid ethyl ester		Inactive	Active
 3,5-Dimethylphenoxyacetic acid	109	Inactive at 20 mg./g.	Active 10
 α-(3,5-Dimethylphenoxy)-propionic acid	119–120	Inactive	Active 0.25
 α-(3,5-Dimethylphenoxy)-*n*-butyric acid	112–114	Inactive	Active 50

TABLE I (*Continued*)

	β-(3,5-Dimethylphenoxy)-*n*-butyric acid ethyl ester		Inactive at 20 mg./g.	Inactive at 20 mg./g.
	2,4-Dimethylphenoxy-acetic acid ethyl ester		Active 0.5	Active 0.25
	α-(2,4-Dimethylphenoxy)-propionic acid ethyl ester		Active 0.5	Active 0.5
	α-(2,4-Dimethylphenoxy)-*n*-butyric acid ethyl ester		Active 20	Active 20
	3,4-Dimethylphenoxyacetic acid	162–163	Active 1	Active 1
	α-(3,4-Dimethylphenoxy)-propionic acid	85–86	Active 1	Active 0.25
	α-(3,4-Dimethylphenoxy)-*n*-butyric acid	73	Active 5	Active 5

tion of the linkage of the phenoxy group to the acid is important. The alpha carbon atom of the acid, for example, appears to be the right place for this linkage. Both xylenoxypropionic and butyric acids are inactive when the beta carbon atom of the acid is linked to the oxygen of the phenoxy group.

An examination of results given in Table I shows that 3,5-xylenoxyacetic acid and its higher homologs have a formative influence but all are inactive for cell elongation. In contrast, 2,5-xylenoxyacetic acid and its higher homologs are active for cell elongation but have no formative influence. In further contrast, when the methyl groups are substituted in the 2,4- and 3,4-positions in the ring of xylenoxy acids, they are active for both cell elongation and formative effects. Compared with halogen substituted phenoxy acids still further differences appear. For example, 2,4-dichlorophenoxyacetic acid is active for both effects, but its higher homologs though active for cell elongation lack the formative influence. If another chlorine atom is substituted making 2,3,5-trichlorophenoxyacetic acid, then the formative influence is lost but the molecule is very active for cell elongation. Many other comparisons and contrasts could be made, but it is still impossible to determine from the appearance of structural formulae what makes a molecule active. It is necessary to make a biological assay to determine whether or not a substance is active.

The formative influence of four xylenoxy acids is shown in Figures 1 and 2. The principal effects appeared on the new leaves or parts of leaves which grew after the plants were treated. The pictures show that some tomato leaves were partially formed when the experiment was started, the tip remaining normal while the remainder of the leaf was modified. Under the influence of these hormone-like chemicals the pattern, texture, and venation of leaves grown were noticeably modified. The change in the position of the veins and the clearing of the veins induced with substituted phenoxy and xylenoxy acids made the leaves resemble those of virus diseased plants (Fig. 1). The effects in both cases are systemic rather than local. Since the causal agent of virus diseases has recently been considered as a chemical compound (2), it seems pertinent to point out these similarities. One difference is that the virus disease can be transmitted by grafting or inoculation while the chemically induced effects cannot.

Another similarity between virus effects and induced effects is the "shoe string" or "fern leaf" pattern of tomato leaves. This response is shown in Figure 2 A and B. These types can be brought out also by genetic crossing and selection. They come true to type when selfed differing in this respect from both virus diseased and chemically induced types. As the chemical influence disappears the plants revert to normal. With few exceptions, virus diseased plants do not recover.

There are many qualitative differences in the various responses which can be induced with xylenoxy acids. For example, xylenoxyacetic acid ef-

FIGURE 1. Modification of leaves induced with vapors of xylenoxy acids. A. *Nicandra* plants. Left, control. Right, exposed to vapors of ethyl 2,4-dimethylxylenoxyacetate when plant was small. B. *Cleome* plants. Left, control. Middle, exposed to vapors of ethyl 2,4-dimethylxylenoxyacetate. Right, exposed to ethyl α-(2,4-dimethylxylenoxy)-propionate when plant was about 5 inches in height. C. *Cleome* leaves from plants shown in "B." Two control leaves on left.

FIGURE 2. Tomato plant parts to show modification of leaves induced with two xylenoxy acids. A. Left, control shoot. Right, response to treatment with lanolin preparation containing 3,5-xylenoxymalonic acid (approximately 50 mg./g.). B. Left, one control leaf. Other leaves from treated shoot above. C. Left, control shoot. Right, response to 3,5-xylenoxypropionic acid (5 mg./g.).

fects differ from xylenoxymalonic acid and the latter from xylenoxypropionic acid (Fig. 2). The responses induced with the more effective xylenoxy acids, 2,4-xylenoxy and 3,4-xylenoxy acids, resembled those of halogen substituted phenoxy acids (4). One difference in particular is that the higher homologs of 2,4-dichlorophenoxyacetic acid do not modify leaves while those of the xylenoxyacetic acid do.

The xylenoxy acids are not as effective for inducing roots or cell elongation as 2,4-dichlorophenoxyacetic acid. However, at 3.2 mg./l. of water, 2,5-xylenoxypropionic acid and 2,5-xylenoxybutyric acid were approximately equal to 0.1 mg./l. of 2,4-dichlorophenoxyacetic acid applied to tomato leaf cuttings. The 3,4-xylenoxy group was active for inducing roots but much less active than the 2,4-xylenoxy group. At 200 to 500 mg./l. of water the xylenoxy acids caused parthenocarpic growth when sprayed on open tomato flowers. This is in contrast with 5 to 10 mg. of 2,4-dichlorophenoxyacetic acid per liter of water which is optimum for this compound when applied to open tomato flowers.

The mono and trimethyl substituted phenoxy acids are now under investigation but not included in this report. The early indications are that the ortho-methylphenoxy series is more active than the corresponding chloro substituted group.

As with other growth substances, any method of application which brought the active compounds into contact with the plants was effective for inducing physiological responses. Aerosol mist made by spraying a growth substance solution (1 mg. of growth substance per cc. of carbon tetrachloride and 1 per cent Sesame oil) onto a hot plate brought about systemic responses similar to those induced with vapors of growth substances. When applied in a greenhouse of 1000 cu. ft. capacity, 100 mg. of α-(2,5-dimethylphenoxy)-propionic acid ethyl ester was sufficient to induce epinasty of leaves and set seedless fruit of tomatoes. The vapors produced by warming the esters on a hot plate were as effective as the aerosol mist, and smaller amounts of the chemicals were required.

SUMMARY

Fourteen xylenoxy acids have been prepared and tested for physiological activity. These substances were effective when applied as aerosols, vapors, sprays, or lanolin preparations.

It has been shown that when these hormone-like substances are applied to plants they induce variable responses. Some have the capacity to induce cell elongation and formative effects while others cause only one of these two responses. The position of the substituted groups in the benzene ring appears to determine in part the activity of the molecule. Linkage between the alpha carbon atom of the acid and the oxygen of the xylenoxy group appeared to be necessary for activity of the molecule.

Plants treated with active xylenoxy acids grew modified leaves differing from the normal in size, shape, pattern, texture, and venation. These modified leaves appeared like those of virus diseases plants. The effects, however, could not be transmitted by grafting or inoculation as is true of virus diseases. After the chemical influence disappeared the treated plants reverted to normal.

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PROLONGING THE LIFE OF CINCHONA POLLEN BY STORAGE UNDER CONTROLLED CONDITIONS OF TEMPERATURE AND HUMIDITY

Norma E. Pfeiffer

Because of the current interest in *Cinchona*, experiments were carried out to determine conditions which would prolong the life span of the pollen. Extension of the interval of viability increases the possibility of breeding desirable hybrids from stocks growing at some distance from each other, of repeating crosses a second season with stored pollen, or of utilizing pollen from desirable trees blooming out of season. It had previously been reported by Feenstra-Sluiter (5) for *C. Ledgeriana* that the duration of viability depended on the humidity of the air since in air saturated with water the quinine pollen appeared to lose speedily all ability to germinate but in dry air retained viability for ten days.

The present experiments using controlled humidities at a favorable temperature, 10° C., in darkness, indicate that *Cinchona* pollen may retain viability in a percentage of the grains (5 to 19 per cent average) for a year in relative humidities of 35 to 50 per cent. On the other hand, storage in a vacuum which has been found advantageous in some other pollens (9, 16) failed to prolong life.

MATERIALS AND METHODS

Most of the pollen used in the tests came from Guatemala through the courtesy of Dr. John R. Shuman. A small amount was sent from Glenn Dale, Md., through the courtesy of Mr. B. Y. Morrison and Mr. W. H. Cowgill who collected when seedlings of *C. Ledgeriana* Moens flowered precociously in October, 1942 (seed sowed in May, 1942).

The Maryland pollen collected December 2 was stored by the collector in gelatin capsules in a stoppered bottle containing dry calcium chloride, and sent through the mails to Yonkers. On arrival December 7, the capsules were immediately transferred to a higher humidity (desiccators with saturated calcium chloride solution) at 10° C. The pollens were in two separate lots, collected from long-styled and short-styled flowers, each from a single plant. These were free from the flower tissues.

Of the two shipments of Guatemala material, the first in September, 1942, consisted of long-styled flowers of "*C. hybrida*" which were cut off in Guatemala, dried over calcium chloride for 18 hours, packed in glass tubes which were evacuated and sealed. These tubes arrived in Yonkers nine days after collection whereupon the pollen from the flowers gave negative results when tested for germination on artificial media.

The second shipment, sent November 3 and arriving in Yonkers the

following day by air express, consisted of branches with inflorescences from a mixed lot of hybrids of *Cinchona*. These branches originally had been cut after dark, placed in water in a refrigerated room (7° to 8° C.) from 8 p.m. to 4 a.m. when they were taken from the cold room, and the leaves were removed and the stem branches cut down to fit a shipping basket. The bases of most of the branches were inserted in wet moss, and the whole wrapped in waxed paper, while a smaller lot was sent without moss, but in waxed paper. The material was in excellent condition upon arrival, especially that in moss. A few twigs wilted in each lot, but there was ample turgid material with buds and fully open flowers. In tests of pollen, there was no significant difference in that from the lot in moss as compared with that lacking moss.

Except for one sample which was tested immediately and a small number of twigs left in water in a refrigerated room (about 10° C.), the larger supply was used for storage under various conditions. The perianth tube with the attached stamens was stored after the terminal portion of the tube had been cut off. Storage at 10° C. in desiccators over saturated solutions of magnesium chloride ($MgCl_2 \cdot 6H_2O$), of calcium chloride ($CaCl_2 \cdot 2H_2O$), of potassium carbonate (K_2CO_3), provided for relative humidities of about 35 per cent, 38 per cent, and 44 per cent (22), while desiccators with appropriate sulphuric acid (H_2SO_4) concentrations, gave 25, 50, and 65 per cent relative humidity (24). Other samples after slight drying were stored in narrow glass tubes which were evacuated, sealed off, and placed at temperatures of 5° C. and −5° C.

Samples removed at intervals were tested for their ability to produce pollen tubes in duplicate tests on an agar-cane sugar medium with and without addition of yeast (2). Feenstra-Sluiter had recommended water or sugar solutions without mentioning concentrations, if the stigma fluid was not used. Concentrations of 7, 10, or 12 per cent sucrose with 1 per cent agar were found suitable in our tests. Analysis of results showed there was no significant difference between results with 10 and 12 per cent sucrose, with or without yeast.

Hanging drops were used on which the pollen was sowed. In Guatemala material, pollen was taken from several flowers, but data for long- and short-styled flowers were recorded separately in only a few cases. The Maryland pollens similarly tested gave separate data for long-styled flowers and short-styled, throughout the storage interval. Pollen tubes developed at room temperatures were counted after three hours in the first tests, later after 18 to 24 hours, and percentage of germination determined. Except where noted, the length of the pollen tubes was at least three times the diameter of the pollen grain and the grains taken into account were normal in appearance.

RESULTS WITH GUATEMALA POLLEN

FRESH POLLEN

Tests were run on pollen directly from the flowers to determine certain points of interest with regard to technique. The data obtained showed variation in the sampling as well as some variation on the media used. A preliminary test with a count of 100 grains each on duplicate sowings on two media on the day the pollen was received gave an average of 46.5 per cent germination after three hours. A more detailed test run two days later from fresh material kept in a cold room gave comparative data for pollen from young anthers not yet cracked, from mature stamens already releasing the pollen, and from older stamens. The average germination after six hours was derived from four counts of between 200 to 300 grains for one mount each from flowers on twigs sent in moss and from the lot not in moss, on two media with different sugar concentrations. From the pollen from the very young anther, the average was 46.6 per cent, from the anther at dehiscence 63.7 per cent, and from the older anthers 57.5 per cent. As a result of this finding, the attempt was made where stored material was used to sow pollen from anthers which had already dehisced.

These sowings had been made without regard to the length of style and stamen in the flowers used. To determine whether the pollen from the two types of flowers differed, germination counts of pollen from short stamens and long stamens in still fresh flowers were made on November 10. More flowers with long styles (short stamens) were available with more abundant pollen, but adequate pollen was present in the short-styled flowers. The average germination for eight mounts for pollen from short stamens (long-styled flowers) was 62 per cent, from short-styled flowers 46 per cent. The counts per mount were based on 300 to 460 pollen grains cultured 16 to 18 hours.

The last test of pollen from flowers held at about 10° C. in darkness was made on November 23, three weeks after the branches were cut. This pollen came from shedding anthers, although the flower buds failed to open under existing conditions. Germination of pollen from long-styled flowers was reduced to 5 per cent, with negative results in pollen from short-styled flowers. The results indicate that viability of the pollen decreases greatly when the flowers are kept under these conditions, with more rapid reduction in that from short-styled flowers.

STORED POLLEN

Storage with controlled humidities at 10° C. Duplicate tests made to determine percentage of germination of the pollen stored in darkness at the different humidities gave fairly uniform results after about two weeks

except that pollen in 25 per cent relative humidity gave slightly lower results, 35 per cent as compared to 41 per cent for magnesium chloride and 46 to 49 per cent in the others. Other tests were made at intervals of approximately 30 to 35 days until the end of March and a final test when the pollen had been stored a year.

Within the first month, there was marked loss of viability at 25 per cent relative humidity (to about 2 per cent germination for pollen from long-styled flowers, negative for pollen from short-styled flowers), definite loss at 65 per cent humidity (16 to 17 per cent germination) with much better retention of germinating power in the other humidities (38 to 45 per cent for long-styled flower pollen, 33 to 40 per cent in short-styled flower pollen).

By December 15, there was practically no germination of pollen at 25 per cent relative humidity, an average of 12 per cent at 65 per cent humidity, but more nearly uniform results in material from the other desiccators. At this time, it began to appear that the K_2CO_3-containing desiccator for some reason to be discussed later was less favorable (32 per cent germination), while those with $MgCl_2$, $CaCl_2$, and H_2SO_4 concentration giving a humidity of 50 per cent were more nearly alike, 52, 44, and 47 per cent germination respectively (Table I A). The differences for less favorable conditions continued to show with a further drop to about 3 per cent for 65 per cent humidity, 22 per cent over K_2CO_3 in January, and negative results for 65 per cent humidity and 19 per cent over K_2CO_3 in March. Meanwhile there was some variation between the other lots which depended greatly on sampling especially since some stamens proved to have sterile pollen. On the whole, results were not markedly different in January, with percentages averaging 26 to 32 per cent for pollen over $MgCl_2$, $CaCl_2$, and H_2SO_4 to give relative humidity of 50 per cent. In February, the two latter were very similar, 36 and 37 per cent, while the germination of pollen from the $MgCl_2$ desiccator averaged 60 per cent, an unusual high point. All averages dropped in the following month except for one previously unopened desiccator (H_2SO_4 to give 35 per cent humidity) which gave a germination of 35 per cent.

At the end of the twelve months of storage, counts were made on two bases, one set depending on tube length being at least three times the spore diameter, a second referring to any tube production. On the former basis germination ran 5, 6, and 9 per cent respectively for humidities controlled by $MgCl_2$, $CaCl_2$, and H_2SO_4 giving 50 per cent relative humidity, with less than 1 per cent for K_2CO_3. The percentages for any tube production, in the same sequence, were 15, 13, 19, and 5 per cent. For statistical analysis, the data for percentages on the basis of tubes at least three times as long as the spore diameter were used throughout.

Due to the wide range in percentages encountered in the data, it was

TABLE I

A. EFFECT OF DIFFERENT RELATIVE HUMIDITIES DURING STORAGE AT 10° C. UPON THE GERMINATION OF POLLEN OF CINCHONA HYBRIDS

Relative humidity	Date	Per cent germination (in duplicate) in various media								
		1% agar + 10% sucrose		1% agar + 10% sucrose + yeast		1% agar + 12% sucrose		1% agar + 12% sucrose + yeast		Av.
35% $MgCl_2$	Dec. 15	54	60	46	49	50	55	50	53	52
	Jan. 20	13	28	29	31	14	39	20	30	26
	Feb. 23	51	55	60	64	67	74	53	56	60
	Mar. 24	19	20	8	22	20	46	20	21	22
	Nov. 9	4	5	3	8	5	6	3	8	5
	Nov. 9*	11	13	21	23	10	13	12	15	15
38% $CaCl_2$	Dec. 15	48	50	47	48	42	43	35	36	44
	Jan. 20	25	28	28	37	17	28	36	40	30
	Feb. 23	23	39	38	47	25	35	34	50	36
	Mar. 24	9	24	21	35	24	27	20	25	23
	Nov. 9	5	9	3	4	5	11	4	4	6
	Nov. 9*	11	18	12	12	10	17	11	12	13
44% K_2CO_3	Dec. 15	29	38	27	31	30	—	31	38	32
	Jan. 20	17	21	23	27	15	25	25	25	22
	Feb. 23	4	—	14	16	9	19	4	6	10
	Mar. 24	17	21	7	8	21	29	20	30	19
	Nov. 9	0.5	1.0	0.7	0.8	0.7	—	0.6	0.7	0.8
	Nov. 9*	5	5	6	7	3	—	3	3	5
50% H_2SO_4	Dec. 15	21	41	41	59	40	60	56	57	47
	Jan. 20	31	33	34	43	24	25	30	35	32
	Feb. 23	34	39	32	33	38	39	36	43	37
	Mar. 24	7	29	6	10	18	—	11	12	13
	Nov. 9	6	10	9	10	8	11	9	11	9
	Nov. 9*	14	19	17	21	17	21	20	22	19

* Percentage of grains producing any tube omitted in Table I B and C.

B. SUMMARY TABLE OF DATA FROM A AFTER CONVERSION TO EQUIVALENT ANGLES

Rel. hum.	Dec. 15	Jan. 20	Feb. 23	Mar. 24	Nov. 9	Totals
35%	370	241	407	221	104	1343
38%	330	263	296	226	108	1223
44%	280	224	151	202	40	897
50%	344	274	299	161	138	1216
Totals	1324	1002	1153	810	390	

† 4DF deducted for the four missing items entered by estimation.

‡ Omitting the 4DF with incomplete data.

C. ANALYSIS OF VARIANCE: DATA FROM A CONVERTED TO EQUIVALENT ANGLES

Source of variation	Degrees of freedom	Sums of squares	Variance
Humidities	3	2,734	911
Dates	4	16,124	4031
Media	3	65	22
Hum. × Dates	12	3,094	258
Hum. × Media	9	182	20
Dates × Media	12	454	48
Hum. × Dates × Media	32†	975	30
Replicates	80	1,497	19
Replicates	76‡	1,399	18

desirable to convert the percentages in the main tables to equivalent angles (21, p. 382–383) before making an analysis of variance for the data under consideration.

The analysis of variance of data for the Guatemala pollen is given in Table I. It is seen that there is a highly significant interaction for humidity$\times$dates, and only the values for K_2CO_3 can be shown to be significantly different at all dates. The consistently low values for the K_2CO_3 series do not appear to be due to the humidity which it was expected to maintain, since the humidity for this solution should have been intermediate between that of $CaCl_2$ and the H_2SO_4 concentration used here. Using the interaction humidity$\times$dates$\times$media as an error term, the least significant difference for a total of 40 items is 100 (15, p. 49, 248) and this indicates in general that $MgCl_2$ gave higher germination counts than either $CaCl_2$ or H_2SO_4. But this margin was due to the high counts for $MgCl_2$ on December 15 and February 23, and further experiment would be necessary to furnish a definite choice among $MgCl_2$, $CaCl_2$, and this H_2SO_4 concentration for use in storing pollen.

The variance for dates is highly significant. The decline in germination power in subsequent months is not a smooth line; the results with regard to changes from each date to the following is different in the lots corresponding to different humidity-controlling solutions. There is some evidence of a lowering in germination values between December 15 and January 20, with a recovery during the next month, but further tests should be made before generalizing on this. It is conceivable that the temperature of the room during the progress of the germination tests was a factor in the January 20 tests; although not apparently differing greatly, it was not controlled. But any possible variation in the conditions for germination on the different dates may not have influenced the count to any considerable extent; thus in the February count the germination was consistently high for the $MgCl_2$ lot and at the same time consistently low for the K_2CO_3 lot. The secondary rise after a preliminary fall in germination counts was obtained for each lot; this rise was considerable in amount for the lot showing the highest average germination ($MgCl_2$), less extensive for the two with intermediate counts ($CaCl_2$ and H_2SO_4), and not occurring until a later date for the lot showing the lowest germination percentage (K_2CO_3). It is a possibility that the same factor is at work as Johnson (8) reports in tree pollens for the time between the eight-month and twelve-month testing, where the latter results had higher values. In *Cinchona*, however, in spite of irregularities, there is a definite loss of vitality from over 50 per cent germination at the start to less than 10 per cent at the end of a year in storage.

Results obtained with the different media indicate that the slight dif-

ference in concentration of sucrose and the presence or absence of yeast had no effect on the germination of *Cinchona* pollen.

Storage in vacuum. Pollen lots stored in small glass tubes which were sealed off after evacuating, were held at 5° C. and −5° C. After nine days, samples gave germination percentages of 8 per cent for 5° C. and 18 per cent for −5° C., indicating great loss of viability in a short time. Another sowing made after 48 days gave less than 1 per cent germination in the few cases where any tubes were formed. These results substantiate those obtained in the first shipment of material in vacuum where room temperature was an additional factor. However, the usually favorable lower temperature also fails to preserve viability in a vacuum.

RESULTS WITH MARYLAND POLLEN

Upon arrival after five days' storage in a gelatin capsule over dry calcium chloride at the variable temperatures of a package in the mail, the samples gave an average of 36 per cent germination for pollen from long-styled flowers and 16 per cent for that from short-styled flowers (Table II A). The pollen was transferred to a desiccator containing saturated calcium chloride solution at 10° C. and tested again after 16 days. There was marked improvement in germination ability to 41 and 39 per cent respectively, which is interpreted as recovery after unfavorable conditions. Subsequent testing after 49 days at the favorable humidity and temperature gave an average of 35 per cent for long-styled flower pollen and 24 per cent for short-styled; after 113 days, 22 and 15 per cent respectively. A final test after 11 months' storage resulted in 7 per cent germination for pollen from long-styled flowers and less than 2 per cent for the short-styled, on the basis of any production of a tube. If the criterion remained a tube three times the diameter of the pollen grain, the germination dropped to 2.5 per cent for long-styled flowers and less than 1 per cent for the short-styled (Table II A).

From the data in Table II, it is apparent that there was a significantly higher percentage of germination for pollen from long-styled flowers. The difference is most marked under what may be considered adverse conditions, as unfavorable relative humidity and temperature previous to the December 7 test and an unfavorable time factor in March and December. The degree of improvement shown upon exposure to better conditions of temperature and humidity is apparently greater in the case of the pollen from short-styled flowers. These results tend to conform with those of other investigators who have found this type of pollen more sensitive than that from long-styled flowers.

As in the case of Guatemala pollen, there is no significant difference resulting from the use of the slightly different sugar concentrations.

TABLE II

A. EFFECT OF STORAGE UPON GERMINATION OF POLLEN OF CINCHONA LEDGERIANA STORED AT 10° C. OVER SATURATED SOLUTION OF CALCIUM CHLORIDE

	Per cent germination—duplicates					
	Date	Medium				Av.
		10% sucrose+1% agar+yeast		12% sucrose+1% agar+yeast		
From long-styled flowers	Dec. 7*	26	37	40	44	35.6
	Dec. 23	42	46	30	44	40.5
	Jan. 25	33	38	34	36	35.2
	Mar. 4	15	17	17	20	17.2
	Mar. 30	19	24	18	28	22.2
	Nov. 9	3	2	3	2	2.5
	Nov. 9**	8	7	6	8	7.2
From short-styled flowers	Dec. 7*	17	21	11	16	16.2
	Dec. 23	35	42	37	40	38.5
	Jan. 25	22	24	21	30	24.2
	Mar. 4	12	19	8	20	14.7
	Mar. 30	15	17	7	10	12.2
	Nov. 9	0	1	0	<1	<1.
	Nov. 9**	<1	2	<1	<1	<2.

* After 5 days over anhydrous calcium chloride, no temperature control.
** Line shows percentage producing any tube.

B. ANALYSIS OF VARIANCE OF DATA FROM A† AFTER CONVERSION TO EQUIVALENT ANGLES

Source of variation	Degrees of freedom	Sums of squares	Variance
Style length	1	409	409
Dates	4	1316	329
Media	1	5	5
Style l. × Dates	4	176	44
Style l. × Media	1	33	33
Dates × Media	4	23	6
Style l. × Dates × Media	4	65	16
Replicates	20	231	12

† Omitting all data for Nov. 9.

DISCUSSION

Scant data are available for pollen longevity in Rubiales, the order to which *Cinchona* belongs. Pfundt (18) reported that *Viburnum opulus* lives 37 days in air dry conditions but 164 days over sulphuric acid, while for *Valeriana dioica* the span of life is increased from 8 to 10 days in air dryness to 17 days at 30 per cent relative humidity and 14 days over sulphuric acid. Without temperature control, neither of these approaches the extension of life seen in the present pollen.

Although the literature on *Cinchona* is voluminous, it is difficult to find

references relating specifically to the pollen or its longevity. Feenstra-Sluiter (5) reported finding no data in the literature but observed speedy loss of viability in air saturated with moisture and retention of viability in dry air for ten days, when pollen collected in small paper sacs was preserved in glass tubes under different humidities. There was also the observation that in dry weather pollen of already withered flowers as a rule had not declined in ability to germinate. Feenstra-Sluiter found ready germination on artificial media using water or sugar solutions. The illustrations indicate fairly uniform size of the pollen grains to show each condition, dry, swollen, or producing tubes.

Our results indicate a maximum germinating power when the anther dehisces, followed by a slight loss soon therafter. Branches held at a favorable temperature in a high humidity room for three weeks bore flowers which gave 5 per cent germination in pollen from long-styled flowers (the more resistant pollen).

In only fair agreement with Feenstra-Sluiter's statement and obviously more specific are our findings for *Cinchona* hybrid pollen with relation to longer storage under controlled humidities at 10° C. in darkness. Lower and higher relative humidities (25 and 65 per cent) prove unfavorable within a short time, with the decrease in viability occurring more rapidly in the drier atmosphere. Intermediate humidities of 35 to 50 per cent were all useful in prolonging life in approximately 20 per cent or more of the pollen for about five months and in 13 to 19 per cent of the grains for twelve months, except where K_2CO_3 solution was used to maintain the desired humidity. Recent tests at room temperature by the quick method devised by Shippy (20) to determine relative humidity were run on the solution used in this desiccator and on K_2CO_3 solutions freshly made up according to the method originally used, with results of 55 per cent relative humidity for the former and 48 to 51 per cent for the latter. The method as used in our test tends to give somewhat higher results than those determined in physical laboratories; for example, saturated $MgCl_2 \cdot 6H_2O$ solution gave 40 to 42 per cent while Obermiller (13) determined 34 per cent relative humidity at 24° to 25° C. The humidity in the desiccator controlled by K_2CO_3 was, however, higher than that found with a freshly made solution, which may have resulted from impurities or from failure to agitate the solution to be sure the surface layer was saturated. Further work would be necessary to be certain of the factors in these results, with the possible desirability of adopting Obermiller's method of making up the solution for consistent maintenance of humidity.

Intermediate humidities (35 to 50 per cent) have been found to be favorable for many pollens (7, 18) especially when used with low temperatures (11, 12, 16, 17) in darkness. It is impossible to draw conclusions on taxonomic or ecological bases or on plant habit with regard to the most

advantageous combination of these factors. Comparing tropical woody forms, one may contrast results with those of pollen of varieties of avocado surviving for intervals of one to five months over calcium chloride at 40° to 59° F. (19) and *Hevea* pollen which survives best at 65 to 75 per cent relative humidity at 6° (4) and pistache pollen giving some germination after one and two years at intermediate humidities at $-1°$ C. (23).

For some temperate zone tree forms Johnson (8) reports that at 2° C. in darkness the following relative humidities are most effective: *Pinus* 50 to 75 per cent, *Picea* 10 to 75 per cent, *Quercus* 25 to 35 per cent. Storage at room temperature when effective requires a lower relative humidity in a narrower range, with lower germination percentages at the end of a year.

Reduced pressure with low temperatures in darkness has been found to be favorable for storage of pollen of citrus forms (9) and lily (16) but less favorable than atmospheric pressure in *Antirrhinum* (10) and barley (1), and sometimes injurious, sometimes favorable for pistache pollen (23). *Cinchona* pollen adds to the number for which it is disadvantageous.

If the production of tubes at least three times the diameter of the pollen grain in length is considered a requisite for functioning in styles, the results of 5, 6, and 9 per cent may be compared to the 6 per cent tube production which Olmo (14) finds a minimum consistent with berry sets equivalent to those from fresh pollen in grapes. In tests on pistache pollen, however, Stone, Jones, and Whitehouse (23) found that fertilized ovules were secured from the use of all the pollens which showed any sign of life in germination; some filled nuts resulted from the use of one- and two-year-old pollens giving only a trace of germination on artificial media. In the case of *Cinchona*, it has not been possible to carry on field tests to determine how the stored pollen functions. It is fair to assume that it will germinate to as great a degree on appropriate stigmas as on artificial media, and it may prove as effective in accomplishing fertilization as stored grape and pistache pollens.

In the Maryland material, an average of 22 per cent tube production for the more resistant pollen from the long-styled flower was found at 38 per cent relative humidity after four months' storage. This dropped to an average of 2.5 per cent (tubes at least three times spore diameter in length) after a year although 7 per cent of the grains were vigorous enough for the emergence of a tube. There is the same possibility here as above that this pollen could result in a good fruit set when used in the field for pollination, but it would be necessary to make field tests to ascertain its adequacy. Comparison of Maryland and Guatemala pollen shows a greater decrease in germination in the former from the four- or five-month interval to about the twelve-month. The reasons for this are not known, although there might be differences due to the first unfavorable storage conditions or to the origin from a precociously blooming seedling rather than from a ma-

turer plant; there is no real knowledge here of the effect of maturity, if any.

With regard to the heteromorphic flowers, Harper (6) reports that *Cinchona* trees with long-styled flowers are more abundant by two or three to one of the short-styled type, that the former are thoroughly pollinated by the afternoon of the first day of opening while the short-styled are relatively undisturbed and hold little or no trace of pollen. His supposition that the latter are pollinated the next day, after the corolla has fallen away, involves a novel technique for enabling pollen to reach the stigma usually deeper in the perianth tube. Regardless of this and the likelihood that many trees are self-sterile as often occurs in dimorphic flowers, it is important to dwell on certain differences in the pollens. In the Maryland samples, there were higher averages for germination of pollen from long-styled flowers at every interval. The greater resistance of this pollen is shown after eleven months of storage when about 7 per cent of the pollen grains germinate as against less than 1 per cent of those from short-styled flowers. This difference as shown by analysis of variance was significant for *C. Ledgeriana*. Not enough data were available for the pollen from Guatemala long- and short-styled flowers to determine the effect in a mixed hybrid lot, although it may be expected to be similar.

Various investigators have called attention to similar differences in pollen from heterostylic plants. Pfundt (18) working on *Primula elatior*, *P. officinalis*, and *Pulmonaria obscura* obtained somewhat higher germination percentages with pollen from long-styled flowers on most media tested. Correns (3) in *Primula acaulis* found the small pollen grains coming from long-styled flowers to be less sensitive and to remain viable longer than the larger grains from short-styled flowers. In *Cinchona*, there is a slight difference in pollen grain size; measured after sowing on an agar-sugar medium, if the dimensions of the pollen from long-styled flowers are considered 100×100, those of the short-styled are 109 to 110×103, while measured in a water mount a greater difference of 117×116 results from speedy swelling.

Correns reported no appreciable difference in length of pollen tubes from the two sets of flowers in *Primula acaulis* on artificial media but tubes from the large grains of short-styled flowers were thicker in the proportion of 4.4 to 3.1. Results with *Cinchona* are inconclusive in regard to length of tube, with the average of the ten longest tubes sometimes higher in one set, sometimes in the other. It seems probable that pollen tube length is too much influenced by factors due to media and conditions in culture to get reliable differences to indicate the potentialities of the two kinds of pollen as to length of tube produced in natural pollination. Obviously in nature, there is a demand for ability to produce a longer tube in the penetration of the long style than in the short.

One other phase, the recovery of pollen from unfavorable conditions

by exposure to better conditions of moisture and temperature, was noted in the Maryland pollen. This has been called "revival" by Nebel and Ruttle in apple pollen (11, 12) and has been noted also in gladiolus pollen (17). In the case of *Cinchona* pollen, although there was not complete loss of germinating power in the dry atmosphere in the five days prior to removal to advantageous conditions, there was a marked increase in germinating ability, especially for pollen from the short-styled flowers, 16 days later. This can be of great importance in the field where it may be necessary to collect and transport under unfavorable conditions although it may be possible to store later under proper storage conditions.

SUMMARY

1. The life of pollen of *Cinchona* may be prolonged to approximately a year by storage at 10° C. with controlled relative humidity of 35 to 50 per cent in darkness.

2. Favorable results were obtained at 10° C. with storage over saturated solutions of magnesium chloride and calcium chloride and over appropriate sulphuric acid concentrations. Pollen over saturated potassium carbonate gave lower germination percentages, for reasons not now clear.

3. At 10° C. and 65 per cent relative humidity, germination drops to about 3 per cent in three months.

4. At 10° C. and 25 per cent relative humidity, germination drops to 2 or 3 per cent in a month, with negative results after six weeks of storage.

5. Reduced pressure either at 5° C. or −5° C. is unfavorable as a storage condition.

6. Pollen from long-styled flowers is significantly higher in germinating power at successive intervals of storage (10° C. over saturated calcium chloride) than is pollen from short-styled flowers. It also survives longer in flowers held at a low temperature.

7. There is some recovery of germinating power upon transfer to favorable conditions of temperature and humidity after too dry conditions at room temperature.

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THE EFFECT OF CARBON DIOXIDE UPON THE CHANGES IN THE SUGAR CONTENT OF CERTAIN VEGETABLES IN COLD STORAGE

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Previous experiments (3, 4, 5) had shown that with the Irish potato the sugar changes that occur when the tubers are placed in cold storage were modified by the presence of carbon dioxide. The rapid rise in reducing sugar which occurs in storage at 5° C. was retarded, or even inhibited for a time, by the presence of CO_2 at a concentration of 5 per cent by volume, while the increase in sucrose which occurs at the same time was accentuated.

The present experiments were undertaken to observe the response of other species to CO_2 under cold storage conditions. The species chosen were such that plant organs other than tubers should be represented, and also that the storage organs should have starch contents different from that of the potato (14 to 20 per cent of the fresh weight). The vegetable tissues chosen were: lima bean, seeds from the green pod, with 9.7 per cent starch; parsnip, roots with 6.8 per cent starch; carrot, roots with only a trace of starch, approximately 0.25 per cent of the fresh weight; and Jerusalem artichoke, tubers with no starch at all, the polysaccharide being inulin.

Each of these vegetables was stored at 5° C. for total periods of 16 to 40 days without CO_2 and with CO_2 added to the atmosphere to give volume percentages of 2.5, 7.5, and 22.5 per cent CO_2. At intervals of 4 to 20 days samples were removed for analysis.

With respect to reducing sugar, it was found that carrot and Jerusalem artichoke resembled potato in that the increase was retarded or inhibited, but with parsnip CO_2 caused an increase in this constituent.

With respect to sucrose, which may either increase during cold storage (potato, parsnip, and Jerusalem artichoke), or decrease (carrot and lima bean), CO_2 had the effect of accelerating any increase and retarding any decrease, except with Jerusalem artichoke in which case the sucrose increase was retarded.

These effects upon the reducing sugar of carrot and upon the sucrose of lima bean were induced with as little as 2.5 per cent CO_2, and with 7.5 per cent CO_2 an effect upon each of these two types of sugar was obtained with each vegetable in the test, except with the reducing sugar of Jerusalem artichoke, in which case a concentration of 22.5 per cent CO_2 was required.

MATERIALS AND METHODS

PLANTS AND SAMPLES

Species. The species used were: carrot, *Daucus carota* L. var. *sativa* DC., variety Red Cored Chantenay; parsnip, *Pastinaca sativa* L. variety Long Hollow Crown; lima bean, *Phaseolus limensis* var. *limenanus* Bailey, variety Fordhook Bush; and Jerusalem artichoke, *Helianthus tuberosus* L. variety Purple.

Samples. At once after harvest the carrot and parsnip roots and the artichoke tubers were washed, spread thinly on a paper-covered surface, dried for an hour or two with an air current from an electric fan, sorted into sizes, and these sizes distributed evenly over the number of samples needed for the test. The lima bean pods were harvested at a stage of maturity such that some of the pods were well beyond the market stage for green-pod lima beans. All over-ripe and immature pods were discarded, and the selected pods were distributed one handful at a time from each picking container to each of the samples to be stored. The numbers of tubers or roots or amount of lima bean pods in each sample are shown in the tables.

The samples were placed in cheesecloth bags, and stored on a shelf in the center of the galvanized iron containers in which the different concentrations of CO_2 were maintained.

CONTAINERS AND CO_2 CONCENTRATIONS

Galvanized iron cylinders, each of 790 liters capacity, were used as containers for maintaining the desired concentrations of CO_2. The large storage room in which these containers were placed was thermostatically controlled and constancy of temperature was maintained by frequent attention. The positions of these containers within the room were adjusted so that the temperatures of all containers were the same, as established by a preliminary period of 30 days before the test started, during which time the temperatures inside of the containers were observed at numerous intervals. Furthermore, the temperature of each CO_2 container was checked each day on which a sample was removed, and that of the control container each day except Sunday.

The CO_2 concentrations were maintained at or near the desired level by gas analysis of a sample of air from each container and by adjustment with CO_2 from a cylinder of the compressed gas. The CO_2 values actually maintained during each test are shown in Table I. It is seen that the desired percentages were approached quite closely, the concentrations found being usually slightly above for the control and the two lower CO_2 concentrations, and slightly below for the highest concentration of CO_2. For convenience in the tables and text, the different lots are referred to as the control lot, and the lots with 2.5, 7.5, and 22.5 per cent CO_2.

The oxygen concentration was maintained at approximately 20 per cent by volume and at no time was the value below 17.0 or above 20.6 per cent for more than one day in any lot. The average value for the oxygen for all lots at all periods was 19.88 per cent by volume. At the start of storage of each vegetable there was a tendency for the oxygen content of the lot with 22.5 per cent CO_2 to fall to values of about 16.5 to 17 per cent during the first day of storage, and after the removal of a sample at each sampling date. This shortage was corrected the next day after the start, and thereafter no difficulty was encountered in maintaining the proper amount of oxygen.

TABLE I

ACCUMULATED WEIGHTED OBSERVED VALUES OF CARBON DIOXIDE CONCENTRATIONS, PER CENT BY VOLUME, FOR THE VARIOUS INTERVALS AND CROPS STORED

Crop stored	Storage interval, days	CO_2 concentrations found when the desired concentrations were:			
		0%	2.5%	7.5%	22.5%
Lima bean	3	0.3	3.3	8.5	22.9
	7	0.3	3.2	7.9	22.5
	11	0.2	3.0	7.8	22.3
	15	0.2	2.8	7.8	22.3
Carrot, field-grown	4	0.2	2.7	7.5	22.3
	8	0.3	2.8	7.5	22.1
	12	0.2	2.8	7.5	22.1
	16	0.2	2.7	7.6	22.1
Parsnip and artichoke	5	0.4	2.8	7.8	22.1
	11	0.3	2.9	7.7	22.0
	20	0.2	2.7	7.6	21.6
	40	0.1	2.6	7.5	21.8
Carrot, greenhouse-grown	5	0.2	2.6	7.6	22.0
	15	0.1	2.6	7.5	22.7
	25	0.1	2.5	7.5	22.6

Note: The crops are listed in the order in which the storage tests were made, parsnip and artichoke being stored simultaneously.

A high humidity was maintained inside of the containers by suspending moist cheesecloths. No undue shrinkage of the stored product resulted, the material being in firm condition at the end of each test.

Moisture determinations were made on the minced tissue of each of the different vegetables at the start, and on all lots at each date of removal from storage. These showed no change in the moisture percentage of each tissue throughout the experiment with the following exception: with Jerusalem artichoke the moistures of the control and the 2.5 per cent CO_2 lots were slightly lower than those of the other lots at the final removal date, 73.9 per cent against 74.9 per cent for 7.5 and 22.5 per cent CO_2, the

average for all lots on all dates being 75.1 per cent. At removal dates other than the final, however, the moistures of the artichoke control and 2.5 per cent CO_2 lots were not different from those of the other lots.

EXTRACTION OF TISSUE

Upon removal from storage the carrot and parsnip roots and artichoke tubers were washed, dried with a towel, and minced by a food grinder. The lima bean pods were shelled, the pods discarded, and the green beans passed through the food grinder. The minced tissue in the case of each vegetable was caught in a broad, shallow pan and was thoroughly mixed by hand. Samples of this minced tissue were taken for a moisture determination, except in the case of the artichoke tissue. The procedure from this stage forward varied with the different tissues.

Carrot. Juice was squeezed through cheesecloth by hand, was centrifuged, mixed, a sample pipetted into a volumetric flask, leaded with neutral lead acetate, made up to volume, centrifuged, deleaded with sodium oxalate, and aliquots taken for the sugar analyses.

Parsnip and lima bean. From the minced tissue, samples of 150 g. of parsnip, or 100 g. of lima bean, were dropped into boiling alcohol in amounts such that the final concentration of alcohol after all of the tissue was added would be approximately 70 per cent by volume. These samples were then set aside until a later date, at which time the alcoholic extract was decanted into a volumetric flask and the tissue was transferred to a Waring Blendor and thoroughly disintegrated; it was transferred to a beaker by means of 70 per cent alcohol (by volume) and heated to boiling on an electric plate, transferred to 250 cc. centrifuge bottles, centrifuged, and the extract decanted into the same volumetric flask; this process was repeated four times, this being found to give complete extraction of sugars. The combined extracts were made up to volume and an aliquot taken after filtering through paper. The alcohol was removed by evaporation on a steam bath, the aqueous extract was transferred to a volumetric flask, leaded, made up to volume, deleaded with sodium oxalate and aliquots taken for the sugar analyses.

Jerusalem artichoke. Samples of 250 g. of the minced tissue were first dropped into boiling alcohol. Later the tissue and alcohol were transferred to tared evaporating dishes on a steam bath. The alcohol was removed by evaporation and the samples were dried to constant weight in a vacuum oven at 70° C. The net weight furnished an estimate of moisture in the original sample. The dried tissue was reduced to a powder with mortar and pestle, was again dried at 70° C. in a vacuum oven. Weighed samples of this powder were transferred to 100 cc. Pyrex centrifuge tubes; the dried tissue was first moistened with a little water, then 30 cc. of water were

added, and the tube was placed in a boiling water bath for three minutes, centrifuged, and the liquid decanted into a volumetric flask. Four further successive extractions were carried out in this manner, it having been found by preliminary tests that this procedure extracted all of the sugars and inulin present in the tissue. The accumulated liquid in the flask was cooled, leaded, made up to the volume, deleaded with sodium oxalate, and aliquots were taken for the sugar analyses.

CHEMICAL ANALYSES

Reducing sugar. Reducing sugar was determined by the Munson and Walker method (1, p. 379), the cuprous oxide being titrated with a potassium permanganate solution which was standardized with sugar solutions of known concentrations at the start of the sugar analyses at each removal interval for each tissue.

Sucrose. As inverting agents both HCl in the cold (1, p. 281) and an invertase solution were tried. With carrot and lima bean there was no difference between the readings obtained with these two agents, and in the case of these two tissues the HCl method was used. With parsnip and Jerusalem artichoke, however, higher readings were obtained with HCl than with invertase, and with these two tissues both hydrolyzing agents were used, the duration of inversion being 16 to 24 hours. The invertase values were used for computing sucrose. The excess of reducing substance shown by the HCl values over those shown by invertase was assumed to be a measure of inulin in the case of Jerusalem artichoke, but with parsnip no assumption is made as to the nature of the substance or substances to which this excess reducing power after hydrolysis is due. The values are shown in Table IV and the effect of CO_2 upon progressive changes in the amounts of this substance or substances are referred to in the text relating to the results with parsnip.

Inulin. The gain in copper reducing power obtained by inverting with HCl in the cold over that with invertase was taken as a measure of inulin, and these values were computed by the method of Scott (8). Tests with a sample of inulin purified by recrystallization until sucrose was absent showed that under these conditions inulin was hydrolyzed completely, and that the amount of inulin taken in the sample for hydrolysis was accounted for quantitatively.

RESULTS

CARROT ROOTS

Two tests were made with carrots, one with roots from plants grown in the field in the summer of 1943, and the other from plants grown in a greenhouse bench from October 26, 1943 to March 9, 1944. The field-grown

roots were fully mature and coarse, while those from the greenhouse, although possibly immature and small, were of good market quality.

Field-Grown Carrot Roots

The results are shown in Table II. The analysis of variance showed that the CO_2 had a significant effect upon both the reducing sugar and the sucrose.

TABLE II

FIELD-GROWN CARROTS: EFFECT OF CO_2 UPON THE SUGAR CONTENT OF ROOTS IN STORAGE AT 5° C.

Days stored	Milligrams per cc. of juice from duplicate samples of 17 roots each							
	Reducing sugar (8.5, 9.8)*				Sucrose (31.7, 27.8)*			
	% CO_2 by volume				% CO_2 by volume			
	0	2.5	7.5	22.5	0	2.5	7.5	22.5
4	11.7 10.2	8.6 7.8	6.9 9.8	9.3 11.3	33.3 28.3	29.8 30.4	36.8 25.7	22.6 29.3
	21.9	16.4	16.7	20.6	61.6	60.2	62.5	51.9
8	16.3 13.5	9.3 12.0	8.5 9.0	9.1 8.0	26.1 30.7	31.0 26.8	32.9 31.3	32.1 32.8
	29.8	21.3	17.5	17.1	56.8	57.8	64.2	64.9
12	18.9 16.5	12.9 12.0	9.5 8.5	9.0 8.6	26.3 22.1	23.9 30.6	32.6 31.9	29.8 29.6
	35.4	24.9	18.0	17.6	48.4	54.5	64.5	59.4
16	20.7 18.6	13.9 13.3	8.7 10.6	8.2 10.1	17.8 23.5	26.9 22.5	30.1 31.9	25.2 28.0
	39.3	27.2	19.3	18.3	41.3	49.4	62.0	53.2
Total	126.4	89.8	71.5	73.6	208.1	221.9	253.2	229.4

* Numbers in parentheses indicate the amount present at the start of storage.

Source	D.F.	Variances	
		Red. sug.	Sucrose
CO_2	3	80.66	44.59
Days	3	19.13	33.48
Interact.	9	7.42	36.68
Samples	16	1.61	11.29

Diff. req. for prob. of .05 and .01

Between CO_2 totals	Red. sugar		Sucrose	
	.05	.01	.05	.01
Of columns	10.7	14.8	28.5	39.3
At each sampling day	5.4	7.4	14.3	19.6

Reducing sugar. The control lot increased in reducing sugar during the cold storage period, and CO_2 retarded this increase. The differences re-

quired for significance are shown at the bottom of Table II, 5.4 being the minimum for odds of 19 to 1 and 7.4 for odds of 99 to 1, in comparisons of the total of two duplicates at each sampling period (6, p. 49, 248). These values were reached by the eighth day of storage, and even 2.5 per cent CO_2 showed values definitely lower than those of the control. The effect of 7.5 and 22.5 per cent CO_2 was to inhibit practically completely this increase in reducing sugar, which in the case of the control lot was approximately 100 per cent. At the end of the experiment the reducing sugar values of the lot receiving 2.5 per cent CO_2 were about 30 per cent lower than those of the control lot.

Sucrose. The control lot showed a continuous decrease in sucrose during cold storage, and, in general, the effect of CO_2 was to retard this decrease. However, there was a high variation between duplicate samples in this test, the error variance being 11.29. The minimum difference required between totals of duplicates on each sampling day, 14.3, was reached only by the 7.5 per cent CO_2 lot on the last two sampling dates. Also, the overall requirement for a difference of 28.5 in the column totals was reached only by the 7.5 lot. This experiment left the sucrose situation somewhat uncertain, and it was for this reason that the greenhouse crop of carrots was grown, in order to determine whether this high variation in sucrose in duplicate samples would be obtained in another lot, and to obtain more conclusive evidence on the effect of CO_2 upon the sucrose content of carrots in cold storage.

Greenhouse-Grown Carrot Roots

The results are shown in Table III. Highly significant effects of CO_2 are shown in the analysis of variance. This lot of carrots showed much less sample variation with respect to sucrose than those referred to in Table II, the error variance being now 4.63 instead of 11.29. The variance for reducing sugar, however, was the same in both lots, 1.67 and 1.61. Considerable difference between these two lots of carrots will be noted in the actual sugar values at the start of cold storage. The greenhouse lot showed much higher reducing sugar values and a much lower sucrose content at the start of storage; in fact the reducing sugar in the greenhouse crop was about as high at the start as the highest value reached by the field-grown lot during cold storage, and also the sucrose was about as low at the start as the value reached by the field-grown roots at the end of the test.

Reducing sugar. Notwithstanding the high initial value for reducing sugar, the control lot increased in this constituent during cold storage. This increase was retarded by CO_2, even the lowest concentration of CO_2, 2.5 per cent, having a marked effect which became evident at least by the fifteenth day of storage. This increase in reducing sugar was inhibited com-

pletely during this 25-day cold storage period by CO_2 in the amounts of 7.5 and 22.5 per cent.

Sucrose. In spite of the initially low sucrose value the control lot showed a decrease in sucrose at least by the fifteenth day. This decrease was re-

TABLE III

GREENHOUSE-GROWN CARROTS: EFFECT OF CO_2 UPON THE SUGAR CONTENT OF ROOTS IN STORAGE AT 5° C.

Days stored	Milligrams per cc. of juice from triplicate samples of 17 roots each							
	Reducing sugar (20.1, 18.6, 19.1)*				Sucrose (23.9, 21.8, 24.7)*			
	% CO_2 by volume				% CO_2 by volume			
	0	2.5	7.5	22.5	0	2.5	7.5	22.5
	24.1	19.7	20.0	17.9	18.3	22.0	24.7	24.3
	19.7	20.7	17.5	20.5	23.5	23.5	24.9	20.6
5	19.9	21.2	19.2	19.4	21.6	22.1	20.5	24.1
	63.7	61.6	56.7	57.8	63.4	67.6	70.1	69.0
	26.3	21.2	19.2	16.2	15.9	20.3	19.3	27.0
	23.6	21.1	18.2	16.6	17.3	19.5	22.2	24.0
15	24.9	22.1	19.2	17.9	21.2	21.0	22.7	23.9
	74.8	64.4	56.6	50.7	54.4	60.8	64.2	74.9
	25.2	23.0	17.6	15.6	19.9	17.6	24.4	27.6
	26.2	22.5	20.7	16.2	16.7	22.3	24.4	24.0
25	25.6	24.9	20.3	18.3	19.7	17.4	20.7	22.3
	77.0	70.4	58.6	50.1	56.3	57.3	69.5	73.9
Total	215.5	196.4	171.9	158.6	174.1	185.7	203.8	217.8

* Numbers in parentheses indicate the amount present at the start of storage.

Source	D.F.	Variances	
		Red. sug.	Sucrose
CO_2	3	71.38	41.48
Days	2	5.60	5.95
Interact.	6	8.20	5.84
Samples	24	1.67	4.63

Diff. req. for prob. of .05 and .01				
Between CO_2 totals	Red. sugar		Sucrose	
	.05	.01	.05	.01
Of columns	11.3	15.3	18.8	25.5
At each sampling day	6.5	8.9	10.8	14.6

tarded by CO_2 at the 22.5 per cent concentration by the fifteenth day, and by both 7.5 and 22.5 per cent CO_2 by the twenty-fifth day.

Discussion of Carrot Results

In some respects these results with carrots resemble those previously obtained with potato tubers (3, 4, 5), but in other respects the situation is

quite different. In both cases CO_2 retarded the increase in reducing sugar, but while CO_2 accelerated the increase in the sucrose content of potato tubers, with carrots there was no increase in sucrose, the effect of CO_2 being merely to retard the rate of decrease. In both cases the sucrose content was higher in the CO_2 lots than in the control, in potato because CO_2 induced the formation of more sucrose, in carrot because CO_2 prevented its loss.

With potato tubers, CO_2 increased the total sugar during storage at 5° C. but with carrots, CO_2 had no effect upon the total sugar. The values in Tables II and III are arranged so that the relative positions of the replicate samples have been retained throughout. Total sugars can be computed for each sample by adding the corresponding values for reducing sugars and sucrose. In this way new tables (not shown here) were prepared listing total sugar values. An analysis of variance of each of these tables showed no significance due either to amounts of CO_2 or to duration of storage. The total sugar was not only the same for all treatments and all dates in each carrot crop, but was nearly the same for the two crops, the average total sugar being 39.8 mg. per cc. of juice for the field-grown carrots and 42.4 for the greenhouse-grown.

This difference in the behavior of potato tuber and carrot root is presumably related to the amount of starch present, potato being high in starch which furnished a source of supply from which sucrose could be formed, while the carrot root is quite low in starch. Platenius (7), after referring to previous articles in which doubts were expressed as to the presence of any starch at all in carrot roots, showed conclusively that a small amount of starch is a normal constituent of carrot roots. The present tests agree with those of Platenius. Starch grains were observed to accumulate in a ring in the glass tubes in which carrot juice was centrifuged. An analysis of the alcohol-extracted residue by the calcium chloride-takadiastase method previously described (2, p. 139) indicated starch to be present in tissue of the field-grown lot of carrots to the extent of 0.2 to 0.3 per cent of the fresh weight.

PARSNIP ROOTS

The effect of CO_2 upon the sugar metabolism of parsnip roots in storage at 5° C. is shown in Table IV. Values for reducing sugar and sucrose are shown in columns 2 to 9, while in columns 10 to 13 are given the values for an, at present, unknown substance or substances, whose existence is inferred because the values for copper reducing substances after hydrolysis by HCl in the cold were higher than those for hydrolysis with invertase. Tests were made in this case with increasing amounts of invertase solution to make certain that enough was being used to obtain the maximum reducing power after inversion. Neither a longer period of exposure to HCl in

TABLE IV

PARSNIP: EFFECT OF CO_2 UPON THE SUGAR CONTENT OF ROOTS IN STORAGE AT 5° C.

Days stored	Milligrams per g. fresh weight, duplicate samples of 19 roots each											
	Reducing sugar (5.0, 4.5)*				Sucrose, by invertase (19.7, 20.5)*				Substance or substances hydrolyzed by HCl in the cold but not by invertase expressed as dextrose (1.3, 2.6)*			
	% CO_2 by volume				% CO_2 by volume				% CO_2 by volume			
	0	2.5	7.5	22.5	0	2.5	7.5	22.5	0	2.5	7.5	22.5
5	5.0	6.0	6.8	8.2	27.7	33.5	34.3	41.1	2.1	1.1	1.6	0.8
	5.7	7.4	6.8	7.6	32.1	31.6	33.4	39.2	1.3	1.6	1.1	0.8
	10.7	13.4	13.6	15.8	59.8	65.1	67.7	80.3	3.4	2.7	2.7	1.6
11	7.0	8.2	9.5	8.8	43.0	48.9	54.3	59.9	2.1	2.4	2.1	0.3
	7.7	7.6	8.9	8.5	47.7	45.7	54.3	53.2	2.6	2.9	1.8	0.8
	14.7	15.8	18.4	17.3	90.7	94.6	108.6	113.1	4.7	5.3	3.9	1.1
20	7.0	7.9	9.8	7.6	47.8	48.1	56.2	64.3	3.4	3.4	2.6	1.6
	7.2	6.3	10.1	9.3	53.2	49.3	66.6	73.6	5.0	2.6	2.4	0.8
	14.2	14.2	19.9	16.9	101.0	97.4	122.8	137.9	8.4	6.0	5.0	2.4
40	5.2	7.4	11.0	12.9	62.3	60.5	67.9	78.1	5.3	4.0	3.2	2.4
	7.2	6.9	10.0	10.6	55.0	52.0	66.0	74.2	6.6	4.7	2.1	2.1
	12.4	14.3	21.0	23.5	117.3	112.5	133.9	152.3	11.9	8.7	5.3	4.5
Total	52.0	57.7	72.9	73.5	368.8	369.6	433.0	483.6	28.4	22.7	16.9	9.6

* Numbers in parentheses indicate the amount present at the start of storage.

Source	D.F.	Variances		
		Red. sugar	Sucrose	Additional substance
CO_2	3	14.71	384.15	8.09
Days	3	7.02	1349.84	9.46
Interact.	9	2.17	18.76	0.92
Samples	16	0.63	14.82	0.29

Diff. req. for prob. of .05 and .01

Between CO_2 totals	Reducing sugar		Sucrose		Additional substance	
	.05	.01	.05	.01	.05	.01
Of columns	6.7	9.3	32.6	45.0	4.6	6.3
At each sampling day	3.4	4.6	16.3	22.5	2.3	3.2

the cold up to 96 hours, nor even heating in boiling water for 10 minutes increased the amount of reducing substances found by inversion with HCl.

This substance, or substances, hereafter referred to for convenience as the "additional substance", was present in small amounts in the control lot at the start, and the amount of it increased during cold storage at 5° C. Unfortunately there were no extra parsnip roots available from this lot to determine whether this fraction would continue to increase during a longer cold storage period.

Reducing sugar. Parsnip roots showed a response to CO_2 in cold storage quite different from that of any other tissue yet tested in this respect: the amount of reducing sugar was *increased* by the CO_2 treatment. These results are shown in Table IV, columns 2 to 5. The control lot showed only a small increase during the 40-day period. The 7.5 and 22.5 per cent CO_2 lots, however, showed increases even by the fifth day of storage, and at the final sampling period gave reducing sugar values about double those at the start, and about 50 per cent higher than those of the control lot.

Sucrose. The control lot increased in sucrose continuously during cold storage, and CO_2 at concentrations of 7.5 and 22.5 per cent accentuated this increase, the gain over the control becoming significant by the eleventh day of storage, and remaining so until the end of the test, at which time the sucrose was about 20 per cent higher in the CO_2 lots than in the control.

"*Additional substance.*" There was a definite increase in this fraction in the control lot during cold storage, and the effect of CO_2 was to retard this increase. Even 2.5 per cent CO_2 was effective in this respect, and 7.5 and 22.5 per cent CO_2 completely inhibited the increase during the entire storage period.

Total sugar. The total sugar in the control lot and in each of the lots in CO_2 increased during cold storage, the increase presumably being made possible by hydrolysis of starch, of which there was an abundance, since this constituent was present to the amount of 6.8 per cent of the fresh weight, as shown by an analysis by the takadiastase method (2, p. 138).

LIMA BEAN SEEDS

Reducing sugar. No reducing sugar was found by this method of sugar analysis in the 70 per cent alcoholic extract of seeds from green pods, either at the start of cold storage, or at any of the sampling intervals during the 15 days of this test, and the CO_2 concentrations used in these experiments were without effect upon this constituent. Since all entries in the table of results were essentially zero the table is omitted from this report.

Sucrose. The sucrose results are shown in Table V. The amount of this constituent decreased in the control lot during storage, and the effect of CO_2 was to retard this decrease. The retardation was definite in the 7.5 and 22.5 per cent CO_2 lots by the eleventh day, and the column totals for

the entire period indicate a difference between the 2.5 per cent CO_2 lot and the control. Starch was present in the lima bean seeds at the start of the tests in the amount of 9.7 per cent of the fresh weight as shown by an analysis by the takadiastase method (2, p. 138).

TABLE V

LIMA BEAN: EFFECT OF CO_2 UPON SUCROSE CONTENT OF SEEDS FROM GREEN PODS IN STORAGE AT 5° C.

Days stored	Milligrams of sucrose per g. of fresh weight of seeds (29.0, 32.4)*—duplicate samples of 2.5 quarts, dry measure			
	% CO_2 by volume			
	0	2.5	7.5	22.5
3	29.6 30.2 59.8	31.0 29.8 60.8	31.9 33.7 65.6	34.1 32.2 66.3
7	27.8 27.8 55.6	30.5 28.0 58.5	29.2 28.2 57.4	28.4 28.2 56.6
11	23.6 25.2 48.8	25.8 25.7 51.5	27.0 26.5 53.5	27.8 27.8 55.6
15	22.4 22.1 44.5	22.8 24.7 47.5	25.7 26.5 52.2	25.8 24.7 50.5
Total	208.7	218.3	228.7	229.0

* Numbers in parentheses indicate the amount present at the start of storage.

Source	D.F.	Variances
CO_2	3	11.74
Days	3	77.87
Interact.	9	4.42
Samples	16	0.76

Differences required

Between CO_2 totals	Probability	
	.05	.01
Of columns	7.4	10.2
At each sampling day	3.7	5.1

Pod condition. Retention of good appearance of the green pods during cold storage at 5° C. was favored by the presence of CO_2. The development of rust-colored blotches which started within a week in the control lot was retarded by CO_2, to an observable extent with 2.5 per cent CO_2 and completely inhibited over the 15 days of storage by CO_2 in the amounts

FIGURE 1. A. Effect of CO_2 upon the pod quality of green lima beans after 15 days in storage at 5° C.: left square, pods from the control lot without CO_2; second from left, pods from lot with 2.5 per cent CO_2; third from left, with 7.5 per cent CO_2; right square, pods from the 22.5 per cent CO_2 lot. B. Effect of CO_2 upon the condition of green pod lima beans after 32 days in storage at 5° C.: left pair, the control lot without CO_2, below showing the condition of the bag on removal from storage, and above the pods after removal from the bag; right pair, the corresponding lot stored in 22.5 per cent CO_2.

of 7.5 and 22.5 per cent CO_2. There were indications that 7.5 per cent had a more favorable effect than 22.5 per cent. The results are shown in Figure 1 A. This effect upon the pod condition was verified by a test with a second picking of lima beans, and results similar to those shown in Figure 1 A

TABLE VI

JERUSALEM ARTICHOKE: EFFECT OF CO_2 UPON THE CONTENT OF SUGARS AND INULIN IN TUBERS IN STORAGE AT 5° C.

Days stored	Milligrams per g. of dry weight, duplicate samples of 20 tubers each											
	Reducing sugar (0.0, 0.0)*				Sucrose, by invertase (339, 353)*				Inulin (365, 372)*			
	% CO_2 by volume				% CO_2 by volume				% CO_2 by volume			
	0	2.5	7.5	22.5	0	2.5	7.5	22.5	0	2.5	7.5	22.5
5	4.3	0.9	3.4	1.7	369	365	336	373	350	316	375	334
	6.4	0.0	0.0	0.0	381	380	357	366	329	326	351	339
	10.7	0.9	3.4	1.7	750	745	693	739	679	642	726	673
11	9.4	9.4	7.2	0.0	454	406	388	375	247	286	315	313
	12.0	1.7	0.0	0.0	457	380	413	367	235	297	274	307
	21.4	11.1	7.2	0.0	911	786	801	742	482	583	589	620
20	8.5	11.5	19.6	3.4	432	435	433	359	269	236	251	353
	12.4	11.1	19.2	0.0	425	422	406	325	255	238	270	327
	20.9	22.6	38.8	3.4	857	857	839	684	524	474	521	680
40	12.8	19.2	18.7	13.2	500	490	452	366	187	174	193	307
	12.0	16.2	19.2	17.5	480	444	474	371	183	224	189	258
	24.8	35.4	37.9	30.7	980	934	926	737	370	398	382	565
Total	77.8	70.0	87.3	35.8	3498	3322	3259	2902	2055	2097	2218	2538

* Numbers in parentheses indicate the amount present at the start of storage.

Source	D.F.	Variances		
		Red. sugar	Sucrose	Inulin
CO_2	3	62.88	7824	5,970
Days	3	310.09	8810	21,174
Interact.	9	36.11	4400	4,649
Samples	16	6.03	230	291

Diff. req. for prob. of .05 and .01

Between CO_2 totals	Red. sugar		Sucrose		Inulin	
	.05	.01	.05	.01	.05	.01
Of columns	20.8	28.7	129	177	145	200
At each sampling day	10.4	14.4	65	89	73	100

were again obtained. In this test the duration was extended to 32 days with 22.5 per cent CO_2, and the effect of CO_2 in preventing spoilage is shown in Figure 1 B.

Vitamin C. Vitamin C determinations were made on the intact seeds at once as soon as they were removed from the lima bean pods at each sampling date by the method previously described (9). There was a gradual decrease in vitamin C in all lots, but no differences were noted between the control and the lots exposed to different amounts of CO_2 during storage, nor were there any differences among the CO_2 lots themselves.

JERUSALEM ARTICHOKE TUBERS

The results with Jerusalem artichoke are shown in Table VI, the analysis of variance indicating a significant effect of CO_2 upon all three constituents for which analyses were made.

Reducing sugar. The control lot showed a continuous gain in reducing sugar during cold storage, this increase beginning at least by the eleventh day. In the presence of 7.5 per cent CO_2 this increase did not occur until between the eleventh and twentieth days, and in 22.5 per cent CO_2 until between the twentieth and fortieth days. The details of the effect of CO_2 are not determinable from these analyses, however, since there was a tendency for CO_2 to induce high amounts of reducing sugar rapidly at the end of the inhibition period. For example, note the rapid increase with the 2.5 and 7.5 per cent CO_2 lots between the eleventh and twentieth days, with the 22.5 per cent CO_2 lot between the twentieth and fortieth days. There was a high variability with respect to reducing sugar between duplicate samples of Jerusalem artichoke, the coefficient of variation (6, p. 22) being 27 per cent, this being more than twice as large as even that for sucrose in the field-grown carrots, and more than three times the average of the other coefficients found in these tests. This high variation in reducing sugar for artichoke tissue was not found for the sucrose or inulin values from the same samples of dried tissue. Perhaps the individual tubers are variable as to the time at which the increase in reducing sugar will start.

Sucrose. Jerusalem artichoke tubers showed a response to CO_2 during cold storage quite different from that obtained with any other tissue yet tested in this respect: the *increase* in sucrose was *retarded*, or even inhibited, by the presence of CO_2. The control lot gained in sucrose during the storage period, the increase at the end being about 40 per cent of the starting value. The presence of 22.5 per cent CO_2, however, completely inhibited this increase during the 40 days of storage, and although 7.5 per cent CO_2 permitted an increase, the over-all effect in comparison with the control lot was one of retardation. The column total for 2.5 per cent CO_2 is significantly lower than that of the control.

Inulin. The control lot showed that inulin was being hydrolyzed during cold storage, this constituent decreasing to about one-half of the starting value in 40 days. This decrease was retarded by the presence of CO_2. The most pronounced effect was obtained with 22.5 per cent CO_2, but the inulin values of the 7.5 per cent CO_2 lot for the total period were higher than those of the control.

SUMMARY

Roots of carrot (*Daucus carota* L. var. *sativa* DC.) and parsnip (*Pastinaca sativa* L.), green pods of lima bean (*Phaseolus limensis* var. *limenanus* Bailey), and tubers of Jerusalem artichoke (*Helianthus tuberosus* L.) were placed in storage at 5° C. in large galvanized iron containers in which the following concentrations of carbon dioxide were maintained: 0, 2.5, 7.5, and 22.5 per cent by volume. At intervals, samples were removed for sugar analyses of the roots, tubers, and the seeds removed from the green pods of lima bean.

Carbon dioxide retarded the increase in reducing sugar which occurred in carrot and Jerusalem artichoke, but it increased the rate in parsnip. No reducing sugar was found in lima bean seeds under any storage condition, or at any stage of storage.

Carbon dioxide accelerated the increase in sucrose which occurred in parsnip, but it retarded the sucrose increase in Jerusalem artichoke. With carrot and lima bean, sucrose decreased during cold storage and CO_2 retarded this decrease.

Parsnip roots were found to contain a substance or substances which was hydrolyzed by HCl in the cold but not by an active invertase solution. This "additional substance" increased in amount in the control lot during cold storage but this increase was inhibited by CO_2.

Hydrolysis of inulin in the Jerusalem artichoke during cold storage was retarded by CO_2.

Retention of good color and condition of the green pods of lima bean during storage at 5° C. was favored by the presence of CO_2.

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THE AEROSOL METHOD OF TREATING PLANTS WITH GROWTH SUBSTANCES

P. W. Zimmerman and A. E. Hitchcock

An increased interest in improved methods of treating plants with chemicals has arisen from the use of insecticidal aerosols. These were first used in a practical way to make a mist or fog by spraying solutions of insecticides on a hot surface (1, 4, 5). An improvement in the method of dispensing insecticides was made by releasing a mixture or solution of the chemical and a liquefied gas from a suitable nozzle (2). One of the most promising gases for the purpose is a refrigerant, dichlorodifluoromethane, known by the trade name "Freon." If the chemical is not soluble in the liquefied gas, acetone or other solvents can be used in the mixture.

The aerosol method appears promising for applying growth substances to plants. Both the hot plate method and the use of liquefied gases can be successfully used (3, 7, 8).

To produce a mist or fog of aerosol in a greenhouse by the former method, a solution of growth substance, carbon tetrachloride, and sesame oil was sprayed from an atomizer upon the hot plate. A suitable concentration was 0.1 per cent growth substance and 1.0 per cent sesame oil in carbon tetrachloride (8). When applied in a greenhouse of 1,000 cubic foot capacity 100 mg. of α-(2,5-dimethylphenoxy)-propionic acid ethyl ester were sufficient to cause epinasty of leaves and set seedless fruit of tomatoes.

Hamner, Schomer, and Goodhue (3) dispersed 240 mg. of β-naphthoxyacetic acid per 1,000 cubic feet of greenhouse. They dissolved 3 g. of β-naphthoxyacetic acid in 27 g. cyclohexanone and placed the solution in a steel cylinder into which 270 g. of dimethyl ether were then forced under pressure. When this mixture was released as an aerosol, it caused parthenocarpy and fruit set of tomato flowers.

The present authors dispersed several kinds of growth substances dissolved in acetone, sesame oil, and a liquefied gas, dichlorodifluoromethane. Three kinds of steel cylinders—sometimes referred to as "bombs" were used for different purposes (Fig. 1). One (Fig. 1 A) having a spring attachment which stops the flow readily was used for making a local application somewhat as an atomizer could be used for spraying. Another cylinder (Fig. 1 B) was suitable for dispensing aerosol growth substances in an entire greenhouse. The latter cylinder held two pounds of the mixture which could be dispensed all at one time or in part to meet concentration requirements. The mist or fog made from this cylinder could be seen in a closed house for two to five hours. A third cylinder manufactured and supplied by the Bridgeport Brass Company of Bridgeport, Connecticut, held one pound of the Freon growth substance mixture and was equipped with an

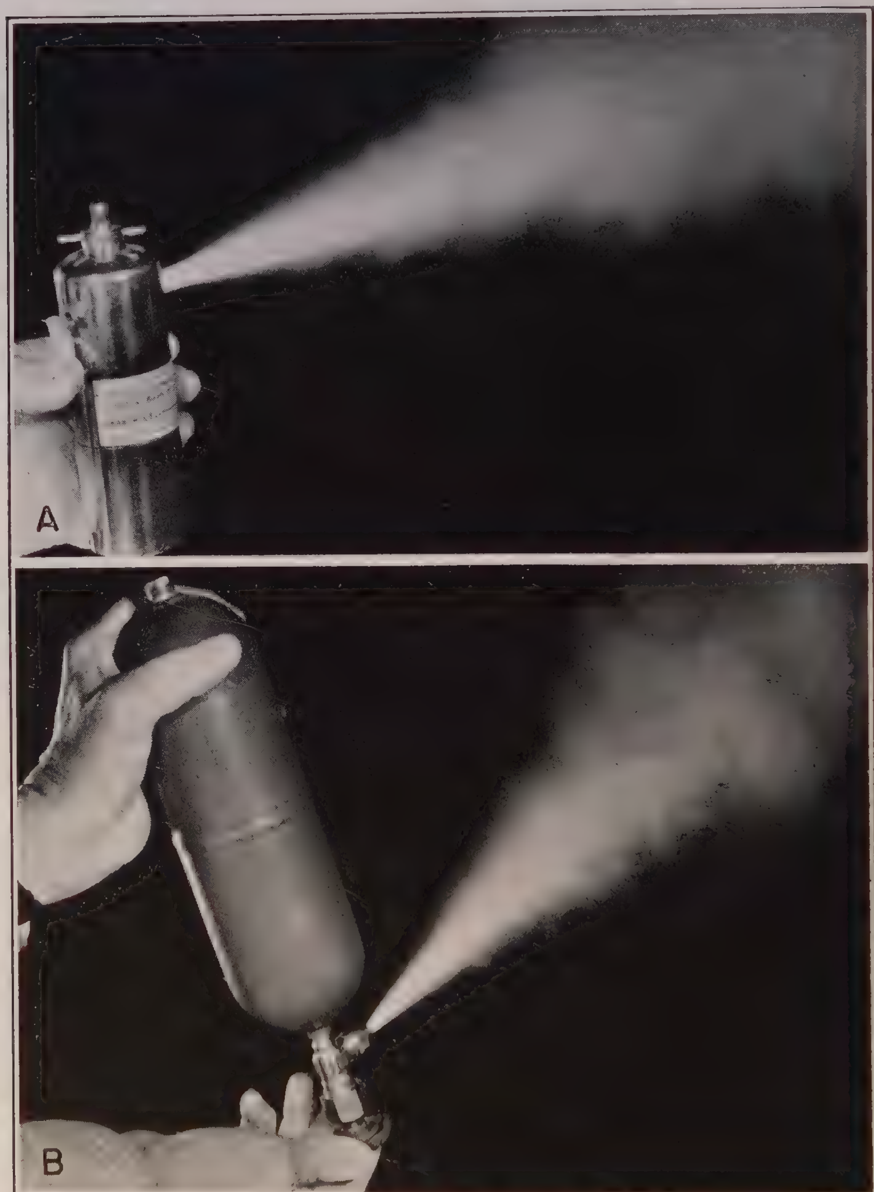

FIGURE 1. Refillable "bomb" dispensers used for applying aerosol growth regulators. A. Cylinder manufactured by the Milwaukee Sprayer Mfg. Co. and filled and supplied for experimental purposes by the Dow Chemical Company of Midland, Michigan. B. Cylinder manufactured by the Pennsylvania Engineering Co., Philadelphia, Pa., and supplied for experimental purposes through the kindness of Mr. W. W. Rhodes of Kinetic Chemicals Inc., Wilmington, Delaware.

FIGURE 2. Tomato flowers and plants to show parthenocarpy and epinasty induced with sprays and aerosols. A. Left, control; right, plant sprayed with a solution of α-(2-chlorophenoxy)-propionic acid (100 mg./l.) from an atomizer. B. Left, two controls not pollinated; right, clusters showing parthenocarpy 5 days after spray solution [1 per cent α-(2-chlorophenoxy)-propionic acid and 3 per cent sesame oil in carbon tetrachloride] from an atomizer was forced around in the air of the greenhouse. C. Left, control; middle, sprayed with 1 per cent 2,5-xylenoxypropionic acid in carbon tetrachloride and 1 per cent sesame oil; right, plant under bell jar with hot plate aerosol from solution in "C."

easily opened and easily closed needle valve. This cylinder was used for treating plants in the field. The aerosol could be directed toward a cluster of flowers, but it was impossible to treat a flower without having some of the chemical touch the leaves.

The aerosol method is a variation of the fine spray method. Figure 2 A shows the results of spraying a water solution of α-(2-chlorophenoxy)-propionic acid (100 mg./l.) over a self sterile tomato plant having a large number of flowers and buds. While some of the spray must have hit the flowers, no special effort was made to treat them. The sprayed plant set fruit abundantly while none set on the non-treated control.

Another solution [carbon tetrachloride 100.0 g., α-(2-chlorophenoxy)-propionic acid 0.1 g., and 3.0 cc. of sesame oil] was dispensed in a greenhouse from an atomizer attached to a compressed oxygen tank. The atomizer was moved from one end of the house to the other as a fine spray was produced. Figure 2 B shows two non-pollinated control flower clusters and two from the greenhouse which was sprayed. A total of 50 mg. of ethyl α-(2-chlorophenoxy)-propionic acid was liberated with the spray in a greenhouse of 2,500 cubic foot capacity. The fine spray, though not pointed directly toward the flowering plants, spread throughout the house and caused fruit set.

In another case the same solution was used to spray a hot plate in the center of the greenhouse to produce a mist. When 50 mg. were dispensed by this method in a greenhouse of 2,500 cubic foot capacity, fruit set was effected on open flowers of tomato plants distributed throughout the house. Figure 2 C shows epinasty induced when the spray from an atomizer was applied directly to a tomato plant. The picture shows also a plant which responded to the mist made by spraying the same solution on a hot plate.

Some of the results of the application of aerosol growth substances dispensed with cylinders are shown in Table I. The cylinders or "bombs" used in the experiments are shown in Figure 1 A and B. In all experiments, except the one indicated with an asterisk, the ethyl ester of α-(2-chlorophenoxy)-propionic acid was dissolved in acetone and placed in the cylinder with sesame oil and Freon. The proportion of growth substance to Freon was not always the same. One-half of a gram to one gram of the ester dissolved in 25 to 50 cc. of acetone to 2 pounds of Freon and 10 to 20 g. (1 to 2 per cent) of sesame oil constituted a satisfactory mixture. The method of flowing the liquefied gas into the evacuated cylinder did not insure always getting the same proportions. Since, however, a known amount of growth substance was first placed in the cylinder and weights taken before and after it was prepared with Freon, known amounts of the active ingredients could be dispensed at any time. There is no good biological method for determining what constitutes the optimum mixture or the amount which should be dispensed per given volume of greenhouse capacity. The tomato

TABLE I

PARTHENOCARPIC DEVELOPMENT OF TOMATO (VARIETY BONNY BEST) OVARIES INDUCED WITH AEROSOL GROWTH SUBSTANCES [ETHYL α-(2-CHLOROPHENOXY)-PROPIONATE] DISPENSED FROM CYLINDERS, AND COMPARED WITH THE SAME CHEMICAL VAPORIZED, AND WITH NON-POLLINATED CONTROLS

Method and amount of active chemical used	Condition of flowers when treated				TotalNo. flowers and buds	TotalNo. fruit set	Per cent set
	Old	Open	Color	Bud			
Aerosol—100 mg. in 2,500 cu. ft. greenhouse	3	14	6	13	36	28	78
Aerosol—450 mg. in 2,500 cu. ft. greenhouse; third cluster on plant	3	32	16	16	67	54	81
Same as above; first cluster on plant	2	12	9	18	41	33	80
Aerosol*—mist directly on flower clusters	2	26	10	17	55	47	85
Aerosol—mist directly on flower cluster	3	6	2	10	21	14	67
Vapor—50 mg. in 2,500 cu. ft. greenhouse	0	9	1	9	19	13	69
Controls—not pollinated	2	11	6	18	37	0	0
Controls—not pollinated	2	7	2	9	20	0	0

* The mixture in the special cylinder with a spring attachment was:

α-(2-Chlorophenoxy)-propionic acid .... 0.1 g.
Freon .... 100.0 g.
Sesame oil .... 2.0 g.

plant, due to its epinastic response, is a fair indicator for the concentration of the active agent. Figure 3 A shows a young tomato plant photographed 18 hours after 100 mg. of ethyl α-(2-chlorophenoxy)-propionate had been liberated as aerosol in a greenhouse of 2,500 cubic foot capacity. The mixture in the cylinder from which the aerosol was dispensed consisted of 500 mg. of ethyl α-(2-chlorophenoxy)-propionate, 25 cc. acetone, 3 cc. sesame oil, and 2 pounds of Freon. The effect of this concentration for fruit set is shown in the first part of Table I. The flowering tomato plants showed some epinasty for three days after exposure to the aerosol, but later made what might be considered complete recovery. There is, however, always danger of killing buds or even the entire plant. This is true also with a spray or pure vapor treatment involving the same chemicals.

Figure 3 B shows a plant photographed 18 hours after 450 mg. of ethyl α-(2-chlorophenoxy)-propionate were dispensed in 2,500 cubic feet. The fruit set for this treatment is shown in the second and third line of data in

FIGURE 3. Tomato plants to show epinasty induced with growth substance aerosols. A. Left, control; right, plant photographed 18 hours after being exposed in a greenhouse of 2,500 cu. ft. capacity to 100 mg. of ethyl α-(2-chlorophenoxy)-propionate which had been liberated as aerosol. B. Left, control; right, plant photographed 18 hours after being exposed in a greenhouse of 2,500 cu. ft. capacity to 450 mg. of ethyl α-(2-chlorophenoxy)-propionate which had been dispensed as aerosol.

Table I. A picture of a cluster of fruit three days after the treatment is shown in Figure 4 A. The mixture in the cylinder consisted of 1.0 g. of ethyl α-(2-chlorophenoxy)-propionate, 25.0 cc. acetone, 3.0 cc. sesame oil, and 1.1 pounds of Freon.

While this concentration of aerosol was very effective for inducing parthenocarpic development of open flowers and well-developed buds (Fig. 4 A), the young flower clusters were damaged. In some cases all the buds of a young cluster abscissed.

One set of data in Table I shows induced fruit set from applying the aerosol directly to the flower cluster from a special cylinder filled and supplied by the Dow Chemical Company (Fig. 1 A). A picture of one of the clusters photographed five days after being treated is shown in Figure 4 B. The mixture in the cylinder consisted of α-(2-chlorophenoxy)-propionic acid ethyl ester 0.1 g., sesame oil 2.0 g., and Freon 100.0 g.

The results of treating only the cluster by this method are very satisfactory, and the rest of the plant is little or not at all affected. This may be the best aerosol method for plants growing out-of-doors. It is similar to applying sprays with an atomizer, but the aerosol method facilitates application. The greatest difficulty comes in preparing the cylinders with the proper mixture.

From these early experiments it appears that the aerosol method of applying growth substances to plants will be useful both in greenhouses and out-of-doors. The full effectiveness of aerosols can, however, be had only in closed greenhouses or other containers, since it is easily blown by wind. The problem of keeping it in contact with the plant for a long enough period might be overcome by using higher concentrations of the active chemical in the mixture for the cylinder. Also, the concentration should be varied according to the comparative activity of the effective chemical. Both acids and esters have been used for aerosols. Since the esters are the more volatile, they should be used where maximum effectiveness is required.

The results obtained through use of aerosol dispensed from the Bridgeport cylinders on plants growing in the field are encouraging. Two concentrations of *o*-chlorophenoxypropionic acid ethyl ester were used. One mixture was made with 20 cc. of acetone, 10 cc. sesame oil, 500 mg. ethyl ester of α-(*o*-chlorophenoxy)-propionic acid, and 1 pound of Freon. The higher concentration was made by doubling the growth substance, the other ingredients remaining the same. Both concentrations were effective for setting fruit on the Marglobe variety of tomatoes. One hundred per cent fruit set occurred on the first clusters of young plants. A total of 24 clusters were treated with the lower concentration and 12 with the higher. The developing fruit showed the same characteristics as those sprayed with a solution of the active chemical. No detrimental effects could be observed from the aerosol coming in contact with the plant except where the dispenser

FIGURE 4. Tomato flower clusters to show induced parthenocarpy. A. Left, control, not pollinated; right, cluster three days after the greenhouse of 2,500 cu. ft. capacity was treated with aerosol growth substance [450 mg. of ethyl α-(2-chlorophenoxy)-propionate in Freon and sesame oil] dispensed from cylinder shown in Figure 1 B. When treated the cluster showed 1 old flower, 3 well open, and 1 bud. B. Left, control, not pollinated; right, cluster 5 days after being treated directly with aerosol growth substance [α-(2-chlorophenoxy)-propionic acid 0.1 g., sesame oil 2.0 g., and Freon 100.0 g.] from cylinder shown in Figure 1 A. The rest of the plant was not intentionally exposed.

was within an inch or less distance from the tissue of the plant. There were a few cases of browning which assumably resulted from rapid cooling where the aerosol was applied to the tissue. Also there appeared to be no damage from retreatment of clusters where some flowers had set fruit while others were still in the bud stage. In fact this might be a good method to increase the size of the fruit and hasten ripening.

Another possibility seems evident for aerosols in the field. That is, insecticides and fungicides, when needed, might be applied in the growth substance aerosol mixture.

Chemicals satisfactory for inducing parthenocarpy and fruit set of tomatoes are listed in Table II. The concentration range was determined with water solutions applied as sprays, but they give some indication of the comparative activity and the amount to be used in aerosols on the

TABLE II

GROWTH SUBSTANCES ACTIVE FOR PARTHENOCARPY, FRUIT SET OF TOMATOES, AND ACTIVITY OR INACTIVITY FOR MODIFICATION OF LEAVES

Phenoxy acids	Effective range of concentrations for parthenocarpy in mg./l. of water	Activity or inactivity for modification of leaves
α-(Phenoxy)-propionic acid	100–200	Active
α-(Phenoxy)-*n*-butyric acid	100–200	Active
o-Chlorophenoxyacetic acid	200–300	Active
α-(*o*-Chlorophenoxy)-propionic acid	25–50	Inactive
α-(*o*-Chlorophenoxy)-*n*-butyric acid	50–200	Inactive
α-(*o*-Methylphenoxy)-propionic acid	50–100	Inactive
α-(*m*-Chlorophenoxy)-propionic acid	50–200	Inactive
α-(*m*-Chlorophenoxy)-*n*-butyric acid	Active	Inactive
p-Chlorophenoxyacetic acid	50–100	Active
α-(*p*-Chlorophenoxy)-propionic acid	50–200	Inactive
α-(*p*-Chlorophenoxy)-*n*-butyric acid	50–200	Inactive
2,4-Dichlorophenoxyacetic acid	5–10	Active
α-(2,4-Dichlorophenoxy)-propionic acid	50–100	Inactive
α-(2,4-Dichlorophenoxy)-*n*-butyric acid	50–100	Inactive
2,4-Dimethylphenoxyacetic acid	300–450	Active
α-(2,4-Dimethylphenoxy)-propionic acid	300–450	Active
2,5-Dichlorophenoxyacetic acid	25–100	Inactive
α-(2,5-Dimethylphenoxy)-propionic acid	100–300	Inactive
α-(2,5-Dimethylphenoxy)-*n*-butyric acid	Active	Inactive
3,4-Dimethylphenoxyacetic acid	Active	Active
α-(3,4-Dimethylphenoxy)-propionic acid	300–500	Active
2,4,5-Trichlorophenoxyacetic acid	25–100	Inactive
α-(2,4,5-Trichlorophenoxy)-propionic acid	10–50	Inactive
α-(2,4,5-Trichlorophenoxy)-*n*-butyric acid	25–100	Inactive
2,4,5-Trimethylphenoxyacetic acid	25–100	Active
2,4,6-Trichlorophenoxyacetic acid	Active	Active
β-(2,4,6-Trichlorophenoxy)-β′-chloro diethyl ether	Active	Active
β-Naphthoxyacetic acid	50–100	Active
β-Naphthoxypropionic acid	50–100	Active
β-Indolebutyric acid	500–1,000	Inactive
2,5-Dichlorobenzoic acid	100–300	Active

basis of those reported herewith. The capacity of some of the chemicals to modify leaves is shown in the last column of the table. This is a matter of considerable importance when the substance is dispensed as aerosol since this would bring the active ingredient in contact with the entire plant. For example, 2,4-dichlorophenoxyacetic acid and its derivatives applied as vapors or aerosols cause a modification in the shape, pattern, and size of leaves (6). The reduction in surface may be pronounced and unsatisfactory. For that reason the chemicals which are inactive or least active for modification of leaves are recommended for practical purposes.

SUMMARY

The aerosol method of treating plants with growth substances has been used successfully for inducing epinasty, parthenocarpy, and fruit set. The hot plate method and cylinders with liquefied gas (Freon) for dispensing aerosol were both effective. When the aerosol growth substances were dispensed in a closed greenhouse the entire plant exposed was caused to respond. By use of a special cylinder having a spring attachment for releasing and stopping the flow of gas, local parts of the plant could be treated without greatly affecting the entire plant. Thirty-one chemicals are listed to show those which cause leaf modifications and their comparative effectiveness for inducing parthenocarpy and seedless fruit of tomatoes.

The first clusters of young tomato plants growing in the field set fruit after being given the growth substance aerosol treatment.

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INDUCING SPORULATION OF ALTERNARIA SOLANI IN CULTURE

S. E. A. McCallan and Shuk Yee Chan

The production of spores in culture by most isolates of *Alternaria solani* (Ell. & Mart.) Jones & Grout is usually very sparse and erratic at the best, as is generally recognized. Rands (13) achieved more or less abundant sporulation by shredding the mycelium and partially drying out the cultures by exposure to sunlight. Likewise, Kunkel (5), by cutting the mycelium or wounding by scraping, obtained many spores. Ramsey and Bailey (12) studied the effects of ultra-violet radiation in detail and found that it definitely stimulated spore production in *Macrosporium tomato* Cke. The irradiation produced greater and more consistent sporulation than that obtained by the mutilation methods of Rands or Kunkel.

In the use of the Early Blight method of evaluating fungicides on tomatoes in the greenhouse (8) it is essential to have an abundant and uniform supply of pathogenic spores of *Alternaria solani*. The method of inducing sporulation if possible should be simple and rapid. An adaptation of the methods of Rands and Kunkel had been used, but as pointed out previously (8, p. 111) the spore yields varied considerably. Accordingly, it appeared desirable to make a further study of the various factors influencing spore production by *Alternaria solani* in culture.

METHODS

Fungus. The Maine strain (isolate No. 52) of *Alternaria solani* previously used and described (8, footnote 5) was employed throughout.

Culturing and scraping. The fungus was cultured on 40 cc. of potato dextrose agar in 9-cm. diameter Petri dishes unless otherwise stated. The agar was seeded in the center with a small piece of mycelium and, after two weeks at 20° C., mycelial growth had covered the entire surface. The growth was then scraped with a straight edged scalpel and the scrapings discarded. With the method previously used (8), the scraped dishes were placed without their covers in moist chambers, the tops of which were held slightly ajar by means of a folded paper towel inserted as a wedge. The chamber was then placed on a south window sill. This former procedure was followed as a standard for comparison. In the case of various present treatments the Petri dish bottoms, immediately after scraping the cultures, were placed with the rims together in pairs and with several thicknesses of paper towel sandwiched between to absorb any excess moisture. Special treatments were given immediately before or after the scraping as indicated, and after 48 hours at 20° C. the sandwiched cultures were exam-

ined for spore production. Most treatments were made on duplicate cultures.

Spore yield. The spores were detached from culture by rubbing with a rubber policeman in the presence of a little water (11), followed by a rinsing from a wash bottle. The resulting suspension was washed through two layers of cheesecloth to remove large pieces of mycelium. The spore suspensions were further diluted as necessary and several spore counts were made in a Fuchs-Rosenthal counting cell, to determine the total yield of spores per culture dish. On the basis of four counts per sample, the mean coefficient of variation for 60 samples examined at different times was 26.5 per cent. Hence a difference of at least 50 per cent would be required for significance between the spore yields of two dishes.

CULTURE MEDIA

In order to determine the possible effects on sporulation, the fungus was cultured on the following media with 1.5 per cent agar: potato dextrose; potato dextrose plus 0.1 per cent orange juice; potato dextrose plus 0.3 per cent orange juice; 0.1 per cent orange juice; potato dextrose plus 0.1 per cent yeast extract; potato infusion; soluble starch 1 per cent; corn meal (Difco); potato slices without agar; tomato leaf infusion; tomato leaf infusion plus 1 per cent dextrose; and water agar without any nutrients. Growth took place on all cultures though differing markedly in appearance; with unscraped mycelium practically no spores were produced on any culture. In the case of the scraped cultures, good spore production (over 100,000 per dish) resulted in the potato dextrose and potato dextrose plus orange juice cultures as well as the tomato leaf infusion containing dextrose. No spores were produced on the potato infusion, the plain tomato leaf infusion, and the potato slices, while the other cultures produced intermediate numbers of spores. Thus it will be seen that, following scraping, none of the cultures tested provided a more favorable medium for spore production than the standard potato dextrose agar.

The presence of proliferation-promoting intercellular hormones in response to injury have been reported by Loofbourow and co-workers (6, 7). These hormones increased the multiplication of yeast cells, and are composed in part of the different vitamin B complexes. Since injury is obviously a factor in stimulating spore production by *A. solani*, a number of vitamins were added to potato dextrose agar. The following vitamins together with the stated concentration in micrograms per liter were used: thiamin 2.5, riboflavin 5, nicotinic acid 5, inositol 2.5, *p*-aminobenzoic acid 2.5, biotin 0.00125, and choline hydrochloride 12. Little difference could be detected in the appearance and growth of the fungus on this culture compared to the potato dextrose check cultures. After scraping and exposure to sunlight on a window sill, no consistent and significant differ-

ence could be determined in the number of spores produced in the vitamin series or check. Various other combinations of the above vitamins and at different concentrations were also tried without success. It is possible that more favorable concentrations of the vitamins might have been obtained; however, the tests with different culture media did not appear promising. Stevens (17) has found that the addition of vitamins was without effect on perithecial production by *Glomerella cingulata*.

HUMIDITY

The favorable response induced by partial drying out of the cultures by sunlight indicated that humidity might be an important factor in spore production. A series of desiccators was prepared containing saturated solutions with an excess of salt necessary to produce relative humidities of 20, 42, 66, and 79 per cent at 20° C. (16). After equilibrium had been attained scraped and unscraped cultures were placed in each humidity chamber. At the end of 48 hours no appreciable number of spores were produced at any humidity; this was confirmed by repeated tests. In one test after six days in the chambers there was some spore production especially at the lower humidities; however, it did not equal that of the check dishes on the window sill. Again it was noted that scraped dishes produced significantly more spores than unscraped dishes. The water content of the media at 20 per cent humidity was 95.7 per cent as compared to that of approximately 97 per cent for the other humidities. Even when cultures were exposed over concentrated sulphuric acid in desiccators and the air partially exhausted, no appreciable spore production occurred even up to six days. Evidently humidity is not a major factor in spore production by *Alternaria solani* in culture.

TEMPERATURE

During the summer months the yield of spores from scraped dishes exposed on the window sill tended to be very erratic and even failed completely at times. In the later case black dots, possibly the primordia of spores, were observed on the apical cells of hyphae from scraped and exposed dishes but no spores developed. This failure indicated a possible temperature effect; accordingly, a series of scraped and unscraped dishes were placed at temperatures ranging in steps of 5° C. from 5° to 35° C. The tests were run three times in duplicate dishes and the results on spore production are given in Table I A. It will be seen that 20° C. was the most favorable temperature for spore production; however, even here no more spores were produced than would be expected from dishes exposed on the window sill under favorable conditions. The failure to produce spores at summer temperatures is confirmed by the high temperature results of Table I. Again it will be noted that cultures not scraped failed to produce spores at any temperature.

TABLE I

EFFECT OF TEMPERATURE ON SPORE PRODUCTION AND VEGETATIVE GROWTH

A. Spore production. Mean yield per dish in thousands

Temperature, ° C.	Culture scraped	Culture not scraped
5	0	0
10	32	0
15	44	0
20	311	0
25	68	0
30	1	0
35	0	0

B. Vegetative growth. Mean diameter of colony in cm. after days indicated

Temperature, ° C.	3	7	11	15	19	23	27	31
5	1.1	1.8	2.3	2.9	3.3	3.6	3.7	3.9
10	1.0	2.5	3.6	4.7	5.6	6.3	6.5	6.9
15	1.0	3.3	5.1	6.6	7.1	7.1	7.1	7.3
20	1.0	4.3	7.5	9+				
25	1.3	5.5	8.4	9+				
30	1.5	4.9	7.0	8.5	9+			
35	1.3	1.7	1.7	1.7	1.7	1.8	1.9	1.9

It seemed of interest also to determine the effect of temperature on vegetative growth. Three days after inoculating the culture dishes, a set of three dishes was placed at each of the above temperatures and the diameter of the colony measured every four days. The results are shown in Table I B, where it will be seen that the optimum range for growth was from 20° to 30° C. At 20° and 25° C. the cultures grew sparsely, had a normal gray color, and the media were a purplish-red; as extremes of temperature were approached the colonies showed a thick growth and darker color and the media remained colorless, indicating a failure of pigment production. No spores were produced at any temperature. After scraping, one set of the cultures was returned to the respective temperatures while the other set was held at 20° C. Only those cultures held originally at 20° or 25° C. produced any spores and here the number was small as would be expected from the data of Table I A.

ULTRA-VIOLET IRRADIATION

The lethal effects of ultra-violet irradiation on different fungus cultures as well as the more limited accounts on the stimulation of spore production have been adequately reviewed by Smith (14). In regard specifically to *Alternaria* and *Macrosporium*, the findings of Ramsey and Bailey (12) on sporulation of *Macrosporium tomato* induced by ultra-violet irradiation have been noted before. Dillon Weston (3), however, obtained sporulation

in *Alternaria solani* not by ultra-violet rays, but by visible light of high intensity. These reports together with the generally favorable effect of exposing scraped cultures to sunlight suggested further studies on irradiation as a means of inducing sporulation in *A. solani*.

A General Electric, small 85-watt capillary mercury arc lamp, type H-3, fitted with an aluminum reflector as described by Arthur and Harvill (1) but equipped with a special ultra-violet transmitting glass bulb, was used for most of these tests.[1] The cultures to be irradiated were exposed individually at a set distance below the center of the lamp. After treatment, unless otherwise noted, the cultures were returned to 20° C. for sporulation.

Time of exposure. Scraped and unscraped cultures were exposed at time intervals ranging from 10 to 2560 seconds and at a distance of 10 cm. below the lamp. Above 640 seconds, the surface mycelium was killed. The test was repeated three times and although the magnitude of spore yield varied significantly from test to test, the trend with time of exposure was constant and mean values for the three tests are given in Figure 1. The individual

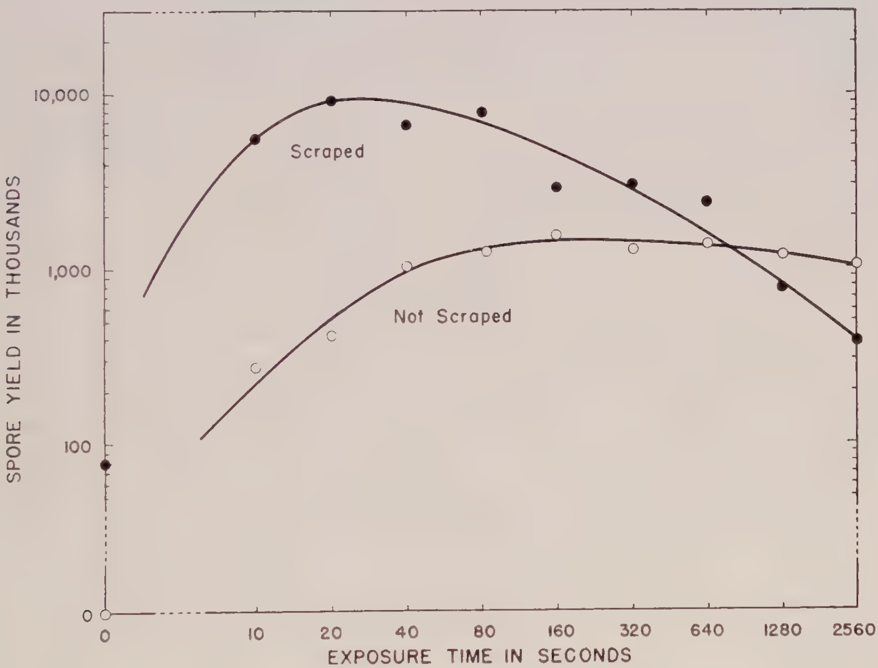

FIGURE 1. Effect of exposure time to ultra-violet irradiation on spore yield from scraped and unscraped cultures of *Alternaria solani*. Note low yields for unexposed checks.

[1] The authors are indebted to Dr. John M. Arthur for kindly supplying the lamp and glass filters, and for making the spectrograms.

yield data were examined by the analysis of variance. The scraped cultures greatly outyielded the unscraped ones and although there was a highly significant test effect, the time of exposure was also significant. The optimum time of exposure for scraped cultures is from about 10 to 80 seconds; however, the unscraped cultures required considerably longer time and even then produced less than one-sixth as many spores. It will be noted that the control scraped cultures produced relatively few spores compared to the irradiated cultures, while the unscraped, non-irradiated controls produced no spores.

Distance from lamp. Scraped replicate dishes were exposed individually at distances of 10, 20, and 40 cm. below the lamp for periods of 5, 10, and 80 seconds. The entire experiment was repeated at a later date and the

TABLE II

EFFECT OF DISTANCE FROM LAMP AND TIME OF EXPOSURE ON SUBSEQUENT SPORULATION OF SCRAPED AND IRRADIATED CULTURES. MEAN SPORE YIELD PER DISH IN THOUSANDS

Distance from lamp in cm.	Time of exposure in seconds		
	5	20	80
10	1540	1750	1470
20	1270	1270	1390
40	1000	1540	1490

mean results are given in Table II. A single degree of freedom analysis (15) showed that at 10 cm. distance the yield was significantly greater than at the longer distances. Hence, hereafter a constant distance of 10 cm. was maintained.

Scraping before or after irradiation. Two tests were made to determine the effect on spore yield of scraping the cultures before or after irradiation. The times of exposure were 10, 20, 40, and 80 seconds. No significant difference could be shown. However, it was decided to continue to scrape before exposing for irradiation.

Humidity and irradiation. Although it was found that humidity made little difference in the spore production of non-irradiated cultures, it was thought that humidity might be a supplementary factor in irradiated cultures. Four tests were made to determine this point, the same desiccator set-up and relative humidities being used as before. The cultures were scraped and irradiated for 20 seconds, then placed at the different humidities for sporulation. The spore yields were high at all humidities and no significant difference could be shown between them; however, the scraped and irradiated control dishes significantly outyielded those in the humidity desiccators.

Temperature and irradiation. Since there was a marked optimum tem-

perature for spore production on non-irradiated cultures even though the yield was relatively low, the effect of temperature on irradiated cultures was also examined. The cultures grown at 20° C. and scraped as usual were irradiated for 20 seconds and placed at the various temperatures. The results of four tests, two of which were on duplicate cultures, are given in Table III. The pronounced optimum temperature at 20° is apparent; this

TABLE III

Effect of Temperature on Sporulation, Following Scraping and Irradiation of Cultures. Spore Yield per Dish in Thousands

Temperature, ° C.	Test No.						Mean production
	1	2	3A	3B	4A	4B	
5	0	0	0	0	0	0	0
10	22	500	240	220	117	96	199
15	141	2,300	6,240	7,200	5,400	3,775	4,176
20	1,101	18,260	11,550	10,830	27,360	14,180	13,880
25	172	7,072	48	53	24	29	1,233
30	0	17	21	28	0	0	11
35	0	0	0	0	0	0	0

agrees well with the data on non-irradiated cultures shown in Table I. However, by irradiating the cultures, the yield at the optimum temperature was increased more than 40-fold, but the difference diminishes as extremes of temperature are reached. The stimulative effect of irradiation is apparently inhibited by high or low temperatures, and the former probably explains the reduction in sporulation by cultures exposed to partial sunlight in midsummer.

Age of cultures. As pointed out above, mycelial growth usually covered the surface of the plate two weeks after inoculation and at this time scraping and irradiation were performed. The effect of age of culture at time of scraping and irradiation on sporulation was tested in three different experiments on duplicate dishes from two to seven weeks old. In some cases the cultures were all inoculated at the same time and irradiated at the appropriate intervals; in others the cultures were inoculated at the appropriate intervals and irradiated at the same time. The results with the two series did not differ, but there was a significant decline in spore production with age as shown in Table IV. Thus two weeks may be considered the approximate optimum for sporulation and has been continued as the standard time.

Cultures usually were examined for sporulation 48 hours after irradiation or other treatment. This time factor was also considered. Replicate dishes 2, 3, and 4 weeks old were scraped and irradiated and a pair examined 2, 3, 5, and 7 days after. No significant difference could be shown in spore yields 2, 3, 5, or 7 days after irradiation. Because of the saving in time, two days was continued as a standard for harvesting the spores.

TABLE IV

EFFECT OF AGE OF CULTURE ON SPORULATION FOLLOWING SCRAPING AND IRRADIATION

Age in weeks	Mean spore yield per dish in thousands
2	9,180
3	4,030
4	1,860
5	860
6	1,230
7	250

Filters. In order to determine the most effective range for inducing sporulation, tests were made using a series of Corning Glass Works filters. Spectrographs of the filters used are given in Figure 2. Filters "A," "B," "C," and "F" were specially made and have been described by Arthur

TABLE V

COMPARISON OF SPORE YIELD FROM SCRAPED CULTURES IRRADIATED WITH OPEN ARC AND THROUGH BLUE PURPLE COREX AND "A" FILTERS FOR 5 TESTS AND 5 TIMES OF EXPOSURE. SPORE YIELD IN 100,000'S

A. Tests Nos. 1 to 5
Spore yield per 3 replicate dishes and 5 exposure times

Filter	1	2	3	4	5	Total
Open arc	1,615	709	2,483	1,676	1,004	7,487
Blue Purple Corex A	1,057	1,112	3,080	4,281	2,872	12,402
"A"	1,223	2,024	2,646	5,341	1,669	12,908
Total	3,895	3,845	8,209	11,303	5,545	32,797

B. Times of Exposure—Seconds
Spore yield per 3 replicate dishes and 5 tests

Filter	10	20	40	80	160	Total
Open arc	1,624	1,518	1,547	1,418	1,380	7,487
Blue Purple Corex A	3,182	3,051	2,304	2,107	1,758	12,402
"A"	2,683	2,930	2,260	2,476	2,559	12,908
Total	7,489	7,499	6,111	6,001	5,697	32,797

C. Analysis of Variance

Source of variation	D.F.	Variance	Significance
Filters	2	119,557	No/Filt.×Tests
Time	4	16,687	No/Filt.×Time
Tests	4	226,216	Sign./Filt.×Tests
Filt.×Time	8	6,623	No/F×T×T
Filt.×Tests	8	54,987	High/F×T×T
Time×Tests	16	10,716	No/F×T×T
Filt.×Time×Tests	32	15,625	High/Repl. dish.
Replicate dishes	150	1,371	

and Newell (2). All of the filters used transmit throughout the visible range excepting the Blue Purple Corex A, the upper limit of which is approximately 460 mμ. The mean results of two tests at exposures of 40 and 80 seconds are shown in Figure 3. The filters had a marked effect and sporulation diminished rapidly as the shorter wave lengths were eliminated. The most effective response was with the Corex Blue Purple and "A" filter and with the open arc. Accordingly a more detailed comparison was made of these three for different times of exposure. In all, five tests were made with three replicated dishes each, and for five different times of exposure ranging from 10 to 160 seconds. The results were examined by an analysis of variance (15) and a summary together with the analysis is given in Table V. The mean values have been plotted also in Figure 3. A large

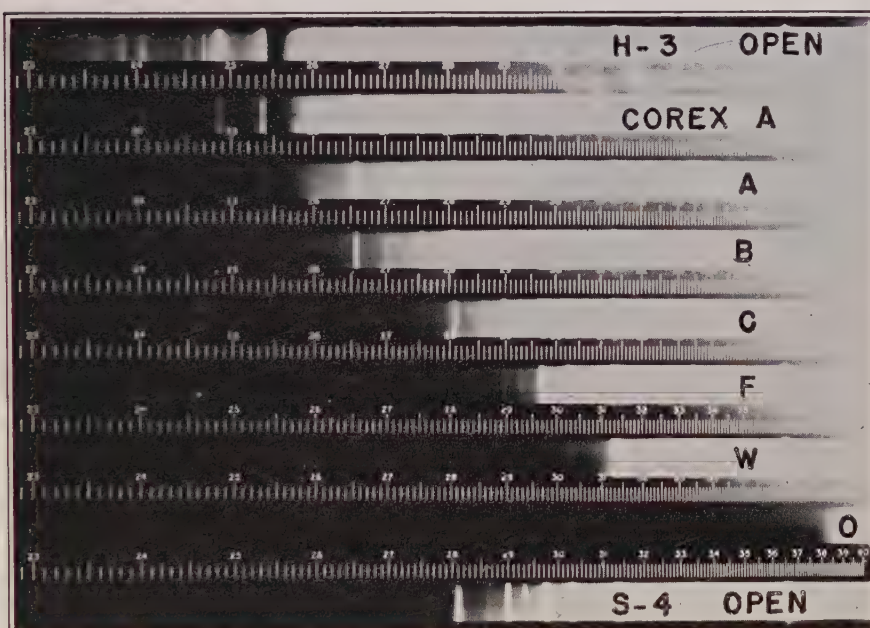

Figure 2. Spectrograms of light transmission with type H-3 lamp, open and with filters "Corex A," "A," "B," "C," "F," window glass, and "Noviol O." Bottom spectrogram type S-4 lamp without filter.

test, or day-to-day variation, will be noted as would be expected from previous results. The two filters and open arc gave very erratic yields in the different tests as may be seen in Table V A. This is presumably due mainly to biological variation associated with different lots of cultures. Such variation has resulted in a highly significant filter × test interaction. Since it is appropriate to use this interaction for error term (9), no significant differ-

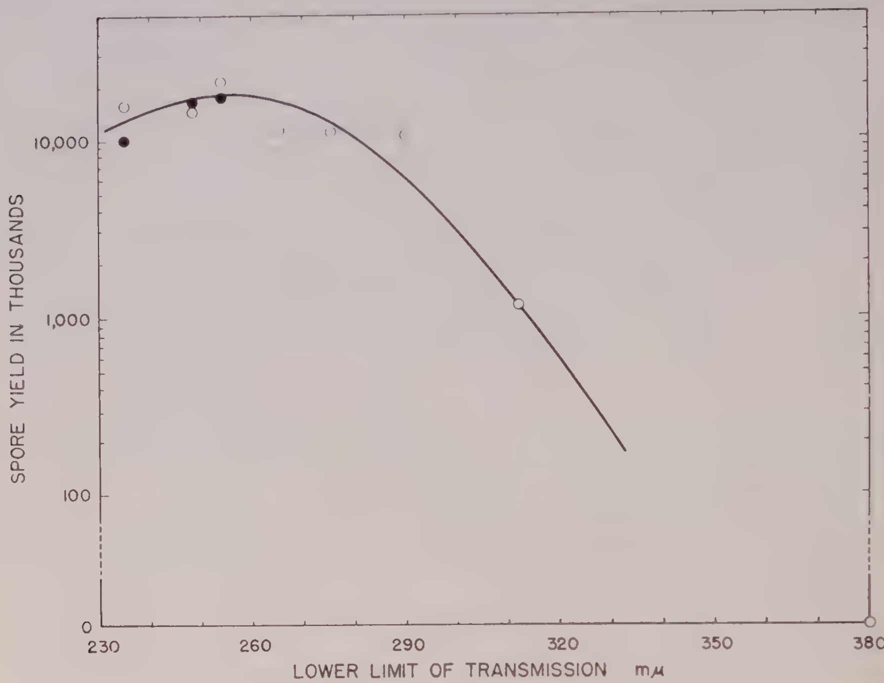

FIGURE 3. Effect of wave length on sporulation of irradiated cultures of *A. solani*. Note o yield at 380 mμ. Solid circles data from Table V.

ence can be shown between the filters and open arc, though a trend showing the favorable effect of the filters is indicated. The yields with the two filters are practically identical, thus showing that radiations above 400 mμ are ineffective in stimulating sporulation since this is the upper limit of transmission for the Blue Purple Corex A filter. Although no significant time of exposure effect was found, a general trend may be observed in Table V B, which closely follows that of Figure 1; that is, the spore yield decreased with time of exposure. This is to be noted especially for the open arc and Blue Purple Corex filter which transmit more of the shorter wave lengths. The variation due to replicate culture dishes was almost negligible. Since the replicates are relatively so uniform, little is to be gained by carrying them. Similar conclusions have been arrived at for replicate spore germination counts (9, 10, 11) and replicate plants in greenhouse tests of fungicides (8).

Ramsey and Bailey (12), using a quartz-mercury arc, obtained maximum sporulation in *Macrosporium tomato* with filters whose lower limits of transmission were between 253 and 280 mμ, and at 40 cm. distance the optimum time of exposure was from 15 to 30 minutes. However, under our conditions maximum sporulation of *Alternaria solani* was obtained from

scraped cultures using filters transmitting somewhat lower wave lengths at shorter distance and for much shorter times of exposure. The saving in time of exposure is a decided advantage where many cultures must be irradiated to produce spores for routine greenhouse tests of fungicides (8).

Other lamps. Since the lamp and bulb used in these studies were not commercially available, a Guard-Ray sun lamp equipped with a General Electric auto-transformer and 100-watt type S-4 lamp was obtained,[2] and its effect compared with that of the former. Spectrograms of the open arcs of both lamps are given in Figure 2. It will be seen that the older lamp transmits more of the lower wave lengths, so that with the addition of Filter C, the transmission is about the same as that of the new lamp. Several comparisons were made at different times on the spore yield resulting from irradiations with the open arc of both lamps and no difference could be shown as would be expected from the data of Figure 3. A lamp capable of transmitting to 249 mμ would in the long run presumably be more effective; however, this lamp without a filter is considered satisfactory.

Infectivity of spores. Spores produced from irradiated cultures were used to inoculate tomato plants under controlled conditions (8) in comparison with those produced by cultures exposed on the window sill. Both lots of spores were equally infective. Several tests were made of the infectivity of spores produced from irradiated two-week-old cultures compared to cultures aged up to five weeks before irradiation. No significant difference in lesion count could be shown.

TIME

A large variation in spore yield from test to test, that is, experiments performed at different times, has been noted above and may be seen in certain of the tables. A similar variation has been found in the LD values for laboratory tests of fungicides (8, 9). In addition, there is some evidence that as the isolate has been maintained in culture over a period of several years there is a reduction in spore yield. This possibly may be associated with the "dual phenomenon" of Hansen (4). However, in any case the spore yields from the cultures irradiated under the favorable standard conditions have continued to be markedly greater than from the non-irradiated cultures produced under the old method.

SUMMARY

1. Undisturbed cultures of most isolates of *Alternaria solani* produce few or no spores. Potato dextrose agar cultures when scraped and placed on a window sill sporulate more or less readily. Other media or the addition of various vitamin B components, in conjunction with scraping and exposure on window sill, were without effect or no better than potato dextrose agar.

[2] George W. Gates & Co., Inc., Franklin Square, Long Island, New York.

2. Maximum vegetative growth and sporulation on scraped cultures occurs at 20° C. At optimum temperature, sporulation tends to increase with decrease in relative humidity. However, in both cases of optimum temperature and humidity the spore yield of checks exposed on the window sill was not attained.

3. Cultures exposed to irradiation from an open mercury arc lamp sporulated abundantly, yielding under favorable conditions approximately 10,000,000 spores per Petri dish culture. Scraped cultures produced a maximum of spores after about 20 seconds' exposure; unscraped cultures required longer and produced less than one-sixth as many spores. Scraping before or after irradiation made no significant difference in spore yield. Maximum spore yields were obtained at 10 cm. distance from the lamp and with two-week-old cultures. Following scraping and irradiation there is a pronounced optimum at 20° C. for greatest sporulation.

4. Irradiation through color glass filters markedly affects sporulation which increases as the wave length transmitted decreases. Greatest sporulation was obtained with filters whose lower limits of transmission ranged from 249 to 254 mμ. Spores produced by irradiated cultures were equal in infective power to those produced by cultures exposed on the window sill.

5. Maximum sporulation by *Alternaria solani* Maine strain 52 may be induced by growing on potato dextrose agar for two weeks at 20° C., scraping, exposing to ultra-violet irradiation for 20 seconds with lamps or filters transmitting to about 250 mμ, and returning to 20° C. for two days.

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A COLORIMETRIC METHOD FOR THE QUANTITATIVE DETERMINATION OF MINUTE AMOUNTS OF TOBACCO-MOSAIC VIRUS AND FOR THE DIFFERENTIATION BETWEEN SOME OF ITS STRAINS[1,2]

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INTRODUCTION

When working with substances available in the pure state in very small amounts from large quantities of the original plant or animal material, quantitative determinations on minute amounts of the purified preparations are often necessary. Likewise, where micro-Kjeldahl determinations are largely relied upon, such as in the quantitative serum-precipitin reaction, amounts of protein too small for accurate analysis by this method are often encountered. Another technic may prove more suitable when the total amount of protein available is less than the limits of error of the micro-Kjeldahl method which are considered to be within the range of 0.06 to 0.13 mg. (9). Of great value for such microdeterminations are the colorimetric methods based upon a definite relation between the concentration of the substance under investigation and the intensity of the color developed under certain conditions either by the substance itself or by one or more of its components. The speed and precision of colorimetric determinations are increased considerably by the use of the photoelectric colorimeter. The Folin phenol reagent in its original form (7) and its modified form (6) is known to produce a characteristic blue color in the presence of tyrosine (7) and tryptophane (1, 8, 19). Cysteine (3, 4) has also been reported to give this color reaction. Knowledge of the fact that proteins containing one or several of the above mentioned amino acids give the characteristic blue color with the phenol reagent has been utilized by several investigators (2, 11, 12, 20) for the development of quantitative methods of determining small amounts of protein. The micromethod suggested by Altschul has been used successfully by Heidelberger and MacPherson (10) for the determination of minute amounts of protein in specific hapten-antibody precipitates.

The purpose of the present study was to investigate the applicability of Altschul's method for rapid determinations of the protein content of minute amounts of purified preparations of the type strain of tobacco-mosaic virus (*Marmor tabaci* H. var. *vulgare* H.) using a Klett-Summerson

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[2] A preliminary report on this work was accepted for presentation before the American Phytopathological Society, New York, New York, December, 1942 (17).

photoelectric colorimeter. The study was extended to include determinations of the colorimetric values obtained by this method for several pure amino acids and also comparisons of the colorimetric values produced under the same conditions by several strains of the tobacco-mosaic virus including the type strain. These comparisons were made for the purpose of determining whether or not this colorimetric method would be suitable for the differentiation of several strains of tobacco-mosaic virus, one of which is known to differ from the others in its tyrosine and tryptophane content (14). Observations were also made on the effect of copper on the development of the color reaction.

MATERIAL AND METHODS

The experiments were performed on ultracentrifuged concentrates of several strains of tobacco-mosaic-virus nucleoprotein, which were kindly supplied by Dr. W. M. Stanley of the Rockefeller Institute for Medical Research. Besides the type strain of tobacco-mosaic virus, the following strains were also used: yellow aucuba (*M. tabaci* H. *aucuba* H.); green aucuba (isolated from the preceding strain); Holmes' rib-grass (*M. tabaci* H. *plantaginis* H.); the masked-symptom strain (*M. tabaci* H. *obscurum* H.); and J 14 D 1 (a derivative of *M. tabaci* H. *lethale* H.). All of the virus preparations possessed the property of inducing disease in susceptible plants. Symptoms characteristic of the type and rib-grass strains of the virus (13) are illustrated in Figure 1. The amount of normal-tobacco-plant protein present in the purified preparations of tobacco-mosaic virus was established from a series of precipitin tests between the virus preparations and antiserum to normal-tobacco-plant protein (5) and was estimated to be less than 1.5 per cent of the total protein. No correction for the normal-plant protein present in the preparations was made subsequently since it seemed unlikely that so small a percentage could appreciably affect the results of these experiments. The preparations which were received in the form of pellets were taken up with an 0.85 per cent solution of sodium chloride and the protein content of each preparation was determined by the micro-Kjeldahl method. A few drops of toluene were added to the contents of each flask and the stock solutions were corked air-tight and stored in a refrigerator for future use. Preliminary to performing the colorimetric determinations, small amounts of the stock solution were withdrawn with a sterile pipette, diluted to a desired concentration, and filtered. Aliquots (20 to 25 cc.) of the diluted solution were used for the determination of its nitrogen concentration by the micro-Kjeldahl method. For the colorimetric determination, aliquots ranging in volume from 0.1 cc. to 2.0 cc. were introduced into tubes and distilled water was added to bring the volume in the tube to 2 cc. This was followed by the addition of 6 cc. of a 12.5 per cent solution of sodium carbonate and 1 cc. of a 0.1 per cent solution of

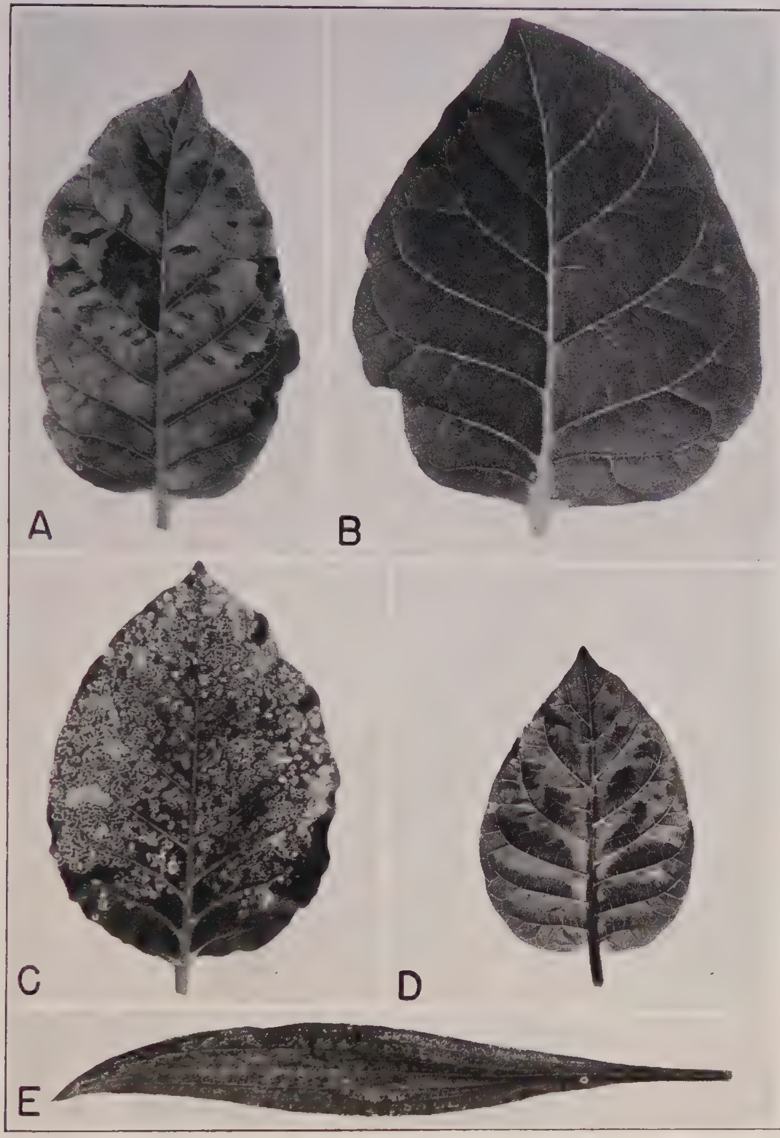

FIGURE 1. Symptoms induced by experimental inoculation of susceptible plants with tobacco-mosaic virus and the rib-grass strain of this virus. A. Chlorotic mottling in Turkish tobacco, *Nicotiana tabacum* L., affected with tobacco-mosaic virus. B. Healthy Turkish tobacco. C. Necrotic-ring lesions on inoculated leaf of Turkish tobacco affected with the rib-grass strain. D. Subsequent chlorotic mottling induced by the rib-grass strain in Turkish tobacco. E. Chlorotic mottling in rib-grass, *Plantago lanceolata* L., affected with the rib-grass strain. (C–E. Holmes, Francis O. Phytopath. **31**: 1089–1098. 1941.)

copper sulphate. The contents of the tubes were thoroughly mixed and allowed to stand for one hour. To obtain consistent results it was found important to keep the virus preparations in contact with the alkaline solution in the presence of copper sulphate for a definite length of time before the addition of the Folin reagent. A series of colorimetric determinations performed in the course of this study in which the only varying factor was this time interval proved that the time factor influenced the colorimetric values developed by the virus solutions with the Folin reagent. For periods of less than 10 to 15 minutes a slight extension in the time interval resulted in a marked increase in the color subsequently developing. Beyond this period the effect of the time interval on the intensity of the color decreased sharply. A time interval of one hour has been adopted arbitrarily in the study because under these conditions the consistency of the results obtained appeared to be greater than with shorter time intervals. At the end of this period 1 cc. of the Folin reagent (6) was added drop by drop with continuous agitation of the tube, the speed of the delivery of the reagent being regulated so as to have the delivery performed in exactly one minute. The tubes were shaken at short intervals and readings were taken in a photoelectric colorimeter 20 minutes after the addition of the reagent. A reagent blank was run with each set of tubes and corrections were made for the colorimetric value of the blank. The majority of tests were made on 10 cc. volumes of the reaction mixture in macro-tubes. When very small amounts of virus were available, volumes of only 3 cc. of reaction mixture were prepared and micro-tubes were used in the photoelectric colorimeter.

CALIBRATION OF THE PHOTOELECTRIC COLORIMETER AND RESULTS OF THE DETERMINATION OF THE CONCENTRATIONS OF TOBACCO-MOSAIC-VIRUS SOLUTIONS

The results of a series of determinations of the colorimetric readings given by the Folin reagent with solutions of known concentrations of several amino acids and of tobacco-mosaic virus are shown in Figures 2 and 3. In most of the colorimetric determinations, including those of tryptophane, tyrosine, and cysteine by means of the Folin reagent, readings on the Klett-Summerson photoelectric colorimeter scale are directly proportional to the concentrations of the substances under investigation, and it is possible to calculate a calibration factor from the colorimetric values of solutions of known concentrations. In case of the virus the relation between the concentration and the colorimetric value has been found to be linear only within very narrow limits of virus concentration. When plotted, this relation for a wide range of concentrations from approximately 0.002 to 0.060 mg. per cc. of the reaction mixture is represented by a line which has a definite curvature (Fig. 3, line A). This line deviates considerably from the regression line calculated by the method of least squares (18) from the

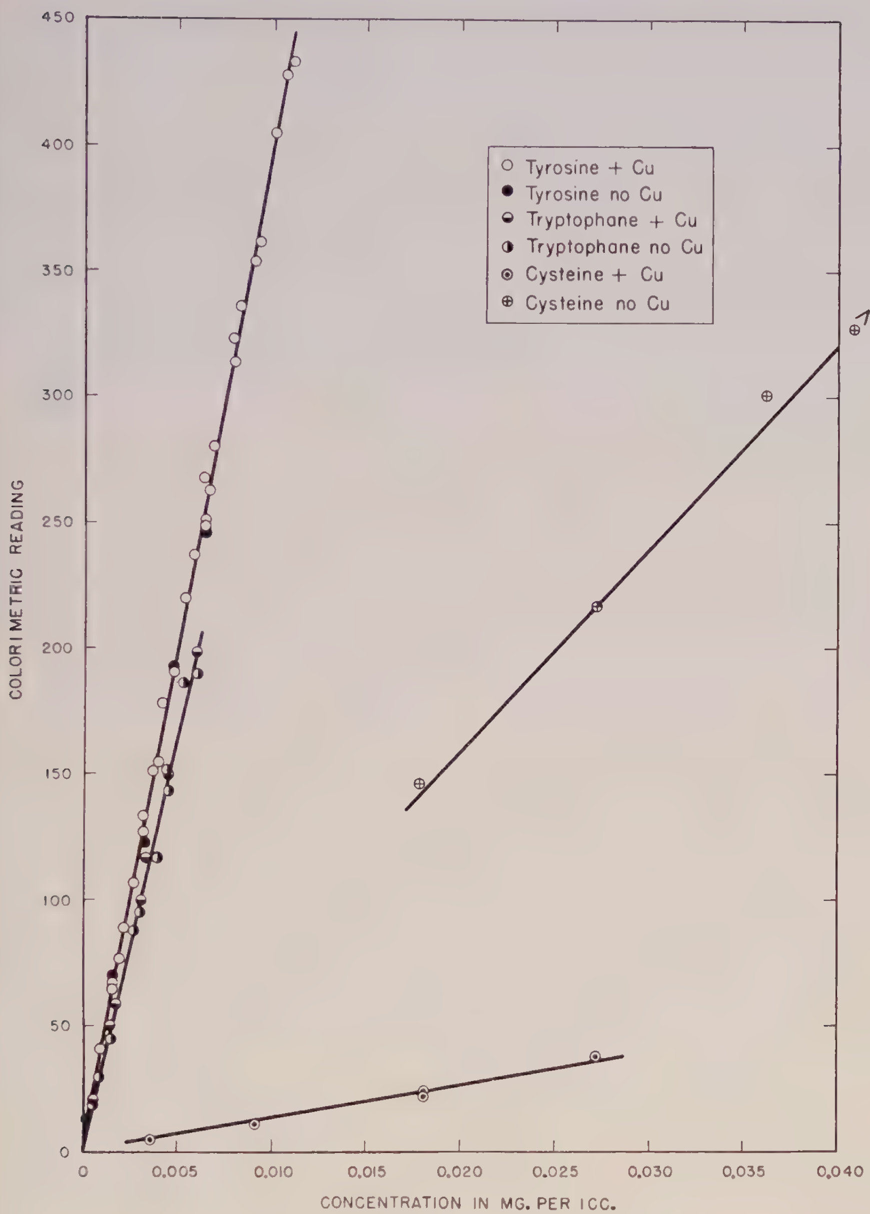

FIGURE 2. Relation between the concentrations of tyrosine, tryptophane, and cysteine, and the colorimetric values produced by these amino acids with the Folin reagent.

experimental data covering the entire range of concentrations used (Fig. 3, line B), the deviations amounting to values as high as 15 units on the colorimetric scale. Due to this lack of direct proportionality between the virus

concentrations and the scale readings, accurate quantitative determinations of virus can not be made by the use of a calibration factor. It appears to be necessary instead to use calibration curves based on a number of determinations of colorimetric readings obtained from a series of solutions of known concentrations.

By plotting the experimental data logarithmically (Fig. 4 A) a good linear relationship is obtained between the logarithms of the colorimetric

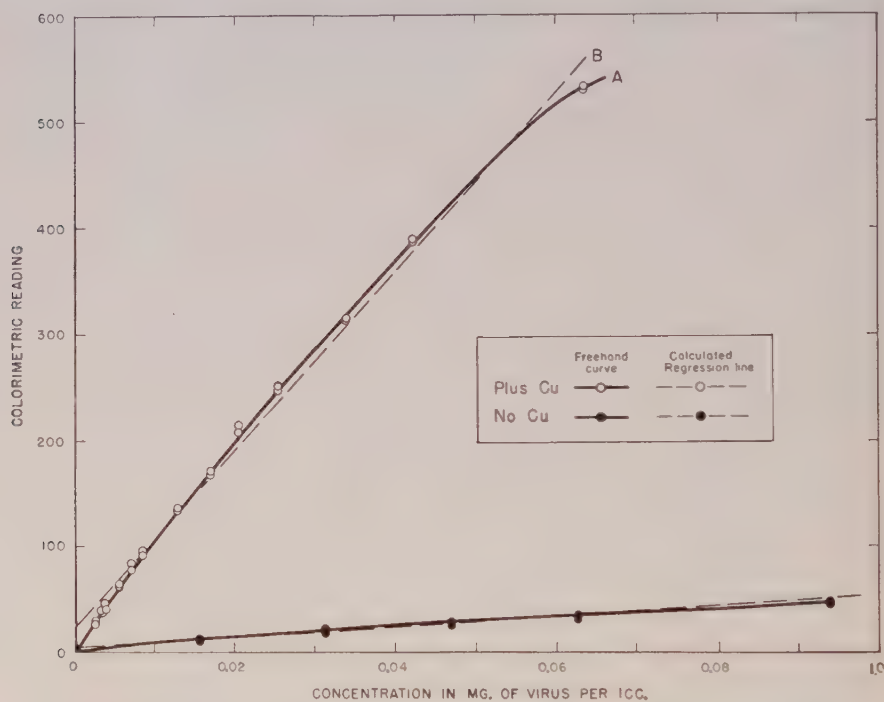

FIGURE 3. Relation between the concentration of tobacco-mosaic virus and the colorimetric values produced by the virus with the Folin reagent. A. Freehand curve. B. Calculated regression line.

readings and the logarithms of the virus concentrations, provided the virus concentrations of the reaction mixture are not lower than about 0.008 mg. per cc. At lower concentrations there is a marked deviation from the logarithmic straight line which fits the data obtained at higher concentrations as shown by the dashed line. The relation for these solutions of low virus content can be expressed by means of another logarithmic regression line calculated by the method of least squares from the data obtained at concentrations below 0.008 mg. per cc. (Fig. 4 A). Evidently while a single logarithmic regression line calculated from data covering the whole range of concentrations used in the experiments can not be used entirely satis-

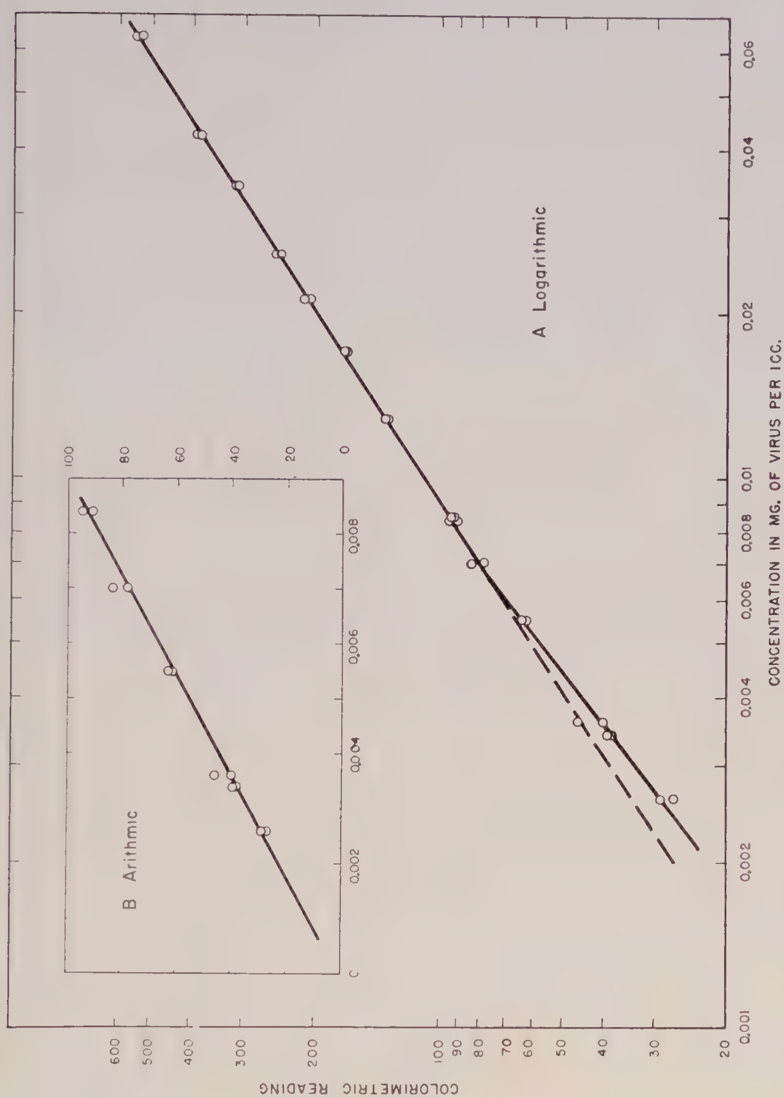

FIGURE 4. Relation between the colorimetric readings and the concentration of tobacco-mosaic virus. A. Logarithmic regression lines calculated separately for concentrations above 0.008 mg. per 1 cc., and for concentrations below 0.008 mg. per 1 cc. The dashed line shows the deviation of the former regression line from the experimental point at the lower concentrations. B. Arithmic regression line calculated for concentrations below 0.008 mg. per 1 cc.

factorily to represent the relation at all these concentrations, a "broken curve" consisting of two logarithmic regression lines calculated separately for concentrations above and below 0.008 mg. per cc. is in good agreement with all the experimental data (Fig. 4 A). For solutions of low virus concentrations within the narrow range of from about 0.002 to 0.008 mg. per cc. the relation between the concentrations and the scale readings can also be expressed by an arithmic regression line (Fig. 4 B) calculated by the method of least squares from data obtained within this range.

The fact that the relation between the virus concentrations and the colorimetric readings can be expressed for the whole range by a combination of two logarithmic regression lines and for the lower range also by an arithmic regression line suggests that it might be more advantageous to employ a combination of two straight lines as calibration lines, instead of using a calibration curve. In order to determine the degree of agreement between the micro-Kjeldahl values for the virus concentrations and the values obtained from the logarithmic and arithmic calibration lines, a number of solutions of different dilutions were prepared from the stock solution and their virus concentrations were determined by the colorimetric method. The amount of virus corresponding to the colorimetric values obtained were read off from both the logarithmic and the arithmic regression lines. Each reading was compared with the results obtained subsequently for the virus concentration by micro-Kjeldahl determinations performed on about 12 to 40 times larger aliquots of the corresponding solutions and the sums of the squares of the deviations were computed (Table I). A comparison of the values of the sums of the squares of the deviations indicates that for virus concentrations of the reaction mixture exceeding 0.008 mg. per cc., readings obtained from the logarithmic regression line calculated for these concentrations give the closest agreement with the Kjeldahl values. For values below 0.008 mg. per cc. a good agreement is obtained by reading them off either the arithmic or the logarithmic regression line, calculated for the lower concentrations. In view of the fact that the differences between the sums of the squares of the deviations obtained by reading the values off these two regression lines are too small to be above the limits of experimental error, for practical purposes it is immaterial whether the values are read off the one or the other regression line. The use of a logarithmic line is preferable for the sake of uniformity in the method of plotting the two regression lines calculated for the two different ranges of concentration. These results establish the fact that the customarily long procedure of making a calibration curve for photoelectro-colorimetric determinations may be eliminated.

Since changes in the intensity of the original light in the photoelectric colorimeter, as well as changes in the reagents used, noticeably affect the colorimetric scale readings, frequent checking of the calibration curves is

TABLE I

COMPARISON OF CONCENTRATIONS OF TOBACCO-MOSAIC VIRUS DETERMINED BY THE MICRO-KJELDAHL METHOD WITH THE VALUES READ FROM CALIBRATION REGRESSION LINES PLOTTED BOTH LOGARITHMICALLY AND ARITHMICALLY

Colorimetric readings	Kjeldahl protein mg./cc.*	Protein mg./cc. from regression line at virus concentration ranges of					
		Logarithmic			Arithmic		
		0.002–0.064	0.008–0.064	0.002–0.008	0.002–0.064	0.008–0.064	0.002–0.008
23	0.0020	0.0019		0.0021	0.0002		0.0020
29	0.0025	0.0024		0.0026	0.0010		0.0025
35	0.0030	0.0029		0.0031	0.0017		0.0030
38	0.0034	0.0032		0.0034	0.0020		0.0033
47	0.0040	0.0041		0.0042	0.0031		0.0041
53	0.0050	0.0047		0.0047	0.0038		0.0047
54	"	0.0047		0.0048	0.0039		0.0048
57	0.0051	0.0056		0.0051	0.0043		0.0052
78	0.0069	0.0072		0.0070	0.0068		0.0070
80	0.0072	0.0074		0.0072	0.0070		0.0072
Sums of squares of deviations		64×10^{-8}		21×10^{-8}	1329×10^{-8}		17×10^{-8}
92	0.0086	0.0086	0.0083		0.0085	0.0069	
97	"	0.0091	0.0088		0.0091	0.0075	
131	0.0125	0.0127	0.0124		0.0131	0.0118	
132	"	0.0128	0.0125		0.0132	0.0119	
167	0.0170	0.0167	0.0164		0.0174	0.0163	
173	"	0.0173	0.0171		0.0181	0.0170	
174	"	0.0174	0.0172		0.0182	0.0171	
176	"	0.0176	0.0174		0.0188	0.0174	
182	"	0.0182	0.0181		0.0192	0.0181	
206	0.0210	0.0209	0.0208		0.0221	0.0211	
208	"	0.0211	0.0211		0.0223	0.0214	
213	"	0.0217	0.0216		0.0229	0.0220	
215	"	0.0220	0.0219		0.0231	0.0222	
241	0.0250	0.0249	0.0250		0.0262	0.0255	
246	"	0.0255	0.0255		0.0268	0.0261	
302	0.0337	0.0321	0.0323		0.0335	0.0331	
325	"	0.0346	0.0351		0.0362	0.0360	
330	"	0.0355	0.0357		0.0368	0.0366	
386	0.0420	0.0406	0.0427		0.0435	0.0436	
383	"	0.0403	0.0423		0.0431	0.0432	
376	0.0424	0.0398	0.0414		0.0423	0.0423	
386	"	0.0405	0.0428		0.0435	0.0436	
Sums of squares of deviations		2612×10^{-8}	1305×10^{-8}		4818×10^{-8}	3040×10^{-8}	

* Average of two or more determinations.

desirable for accurate work. Complete recalibration of the apparatus is evidently necessary whenever the experimental conditions are changed by replacement of one light filter with another presumably identical filter, by replacement of an old electric light bulb with a new one, or by the use of fresh reagents. The photoelectric colorimeter can be calibrated rapidly

for the tobacco-mosaic virus by determining in duplicates the colorimetric readings of as few as three solutions of known concentrations, one of the solutions having a concentration of virus nucleoprotein in the reaction mixture of about 0.002 to 0.003 mg. per cc., the second of 0.007 to 0.008 mg. per cc., and the third of 0.020 to 0.060 mg. per cc. By plotting the results of the colorimetric determinations on logarithmic paper and drawing a straight line through the first two points, the calibration line for solutions of low virus concentration is obtained. A straight line drawn through the second and the third points serves as a calibration line for solutions of concentrations higher than 0.008 mg. per cc. of reaction mixture. The introduction of a third known point on each of these two logarithmic lines would be valuable for checking the correctness of these calibration lines. The virus concentration of an unknown solution can readily be determined subsequently by obtaining its colorimetric value by the usual procedure and then reading the concentration off from the appropriate calibration line. (See Fig. 4.)

Determinations were made on reaction mixtures ranging in concentration from 0.002 to 0.060 mg. per cc. which corresponds to a total amount of from 0.020 to 0.600 mg. of the virus in 10 cc. of the reaction mixture. By using micro-tubes for the colorimetric readings and a total volume of 3 cc. of reaction mixture, it is possible to make determinations on quantities as small as about 0.006 mg. of tobacco-mosaic virus. The precision of this method has been found to be about 7 per cent for reaction mixtures of concentrations ranging from 0.002 to 0.010 mg. per cc., and about 5 per cent for the higher concentrations used.

COMPARISON OF THE COLORIMETRIC REACTIONS OF TYROSINE, TRYPTOPHANE, AND CYSTEINE WITH THE REACTION OF TOBACCO-MOSAIC VIRUS

In view of the difference in the molecular weights of tyrosine and tryptophane, the colorimetric values given by a definite weight of tryptophane with the Folin reagent should theoretically be equal to 88.7 per cent of the colorimetric value given by an equal weight of tyrosine treated in a similar way. In these experiments the colorimetric values given by tryptophane amounted to only 83.3 per cent of those given by tyrosine (Fig. 2). This discrepancy between the theoretical and experimental results is attributed to the instability of the Folin reagent at alkaline pH values (7) as well as to the difference in the speed of the reaction of the two amino acids (6). The Folin reagent can react with the amino acids only in strongly alkaline solutions, the alkalinity of these solutions being such as to produce a rapid decomposition of the reagent. The reaction between tyrosine and the Folin reagent proceeds rapidly and all the tyrosine presumably reacts before the decomposition of the reagent is completed. Tryptophane, how-

ever, reacts slowly, and unless there is a large excess of the Folin reagent, the latter becomes decomposed before the reaction with tryptophane has reached completion. When an excess of reagents is avoided by diluting the Folin reagent, only a small percentage of the tryptophane present contributes to the color given by a mixture of tryptophane and tyrosine, most of the colorimetric value being due to the tyrosine present. Weight for weight the colorimetric values produced by cysteine (Fig. 2) are equal to only three per cent of the values given under similar conditions by tyrosine, provided the Folin reaction proceeds in the presence of more than mere traces of copper ions. In the absence of copper in the reaction mixture, the values given by cysteine are equal to 18 per cent of those contributed by tyrosine. The presence or absence of copper in the reaction mixture has no effect on the color developed with the Folin reagent by either tyrosine or tryptophane.

The tobacco-mosaic virus has been found to contain 3.9 per cent tyrosine, 4.5 per cent tryptophane (16), and 0.7 per cent cysteine (15). For example 0.500 mg. of tobacco-mosaic virus contains 0.0195 mg. of tyrosine, 0.0225 mg. of tryptophane, and 0.0035 mg. of cysteine, and would be expected to give a colorimetric reading of about 150 (Fig. 2). The actual reading given by 0.500 mg. of tobacco-mosaic virus with the Folin reagent is equal to 440 scale readings (Fig. 3). Under the conditions of these experiments, the tyrosine and tryptophane thus contribute not more than one-third of the colorimetric value given by tobacco-mosaic virus with the Folin reagent in the presence of copper, the other two-thirds being furnished evidently by one or more other compounds present in tobacco-mosaic virus and capable of reacting with the Folin reagent. Observations of a similar nature have been made by Herriott (11, 12), who reported that purified gelatin, which is known to contain little or no tyrosine or tryptophane, produces marked colorimetric values with the Folin reagent in the presence of copper. Since the amount of tyrosine and tryptophane in gelatin would be too small to account for the color developed, Herriott suggested the possibility of the existence in the protein of amino acids or other components which in the free state do not react with the Folin reagent, but might react when in combination with other amino acids. In the later paper Herriott (12) reported that some peptides as well as some substances different in nature from amino acids produce a color with the Folin reagent only in the presence of copper.

Contrary to the reactions of tyrosine and of tryptophane, the reaction of the tobacco-mosaic virus with the Folin phenol reagent is greatly influenced by the presence of copper ions (Fig. 3). A change in the usual method of preparation of the reaction mixture whereby 1 cc. of distilled water is substituted for 1 cc. of a 0.1 per cent copper sulphate solution results in a 14-fold decrease in the colorimetric scale readings. While in the

presence of copper ions only one-third of the colorimetric values could be accounted for by the presence of tyrosine and tryptophane in the tobacco-mosaic virus, two-thirds being due apparently to the presence of some other substances in the virus nucleoprotein, in the absence of copper ions these other substances evidently do not contribute to the colorimetric value of the reaction mixture. Moreover, the colorimetric values produced by tobacco-mosaic virus with the phenol reagent in the absence of copper ions are much lower than the theoretical values calculated on the basis of the tyrosine, tryptophane, and cysteine content of the virus.

COMPARISON OF THE COLORIMETRIC VALUES GIVEN BY DIFFERENT STRAINS OF TOBACCO-MOSAIC VIRUS WITH THE FOLIN REAGENT

The modified Altschul method for the determination of small amounts of protein was applied to six different strains of tobacco-mosaic virus. One of these strains, Holmes' rib-grass, is known (14, 15, 16) to differ from the type strain in its content of several amino acids, the type strain containing 3.8 per cent tyrosine, 4.5 per cent tryptophane, 0.7 per cent cysteine, no methionine, and no histidine, while the rib-grass strain contains respectively 6.4, 3.5, 0.7, 2, and 0.55 per cent of these amino acids. The other four strains of tobacco-mosaic virus, namely yellow aucuba, green aucuba, Holmes' masked, and J 14 D 1, differ very little from the type strain in their amino acid content.

Special care was taken in comparing the colorimetric values given by the rib-grass strain with those of the type strain: the reaction mixtures were adjusted carefully to similar concentrations of the two viruses, the reactions were allowed to proceed simultaneously and readings of the colorimetric values of the one and of the other reaction mixture were taken alternately. Under these conditions any difference in the colorimetric values developed by the two strains could be attributed entirely to the difference in the properties of the two substances, and not to external influencing factors. A close examination of the results obtained reveals that at practically all the concentrations used the colorimetric readings obtained for the rib-grass strain were slightly higher than those resulting for the type strain. The differences obtained between the type strain and the other four strains were small and not consistent (Fig. 5 A). A statistical analysis of the results was made, logarithmic regression lines were calculated for each virus strain and the differences between the values obtained for the type strain and each of the other strains were calculated for several different concentrations of the viruses. The values of t, i.e., the quantities representing the differences between the sample and the population means expressed in terms of standard error (18), are given in Table II for each strain. The table also includes the values of t required for the difference to

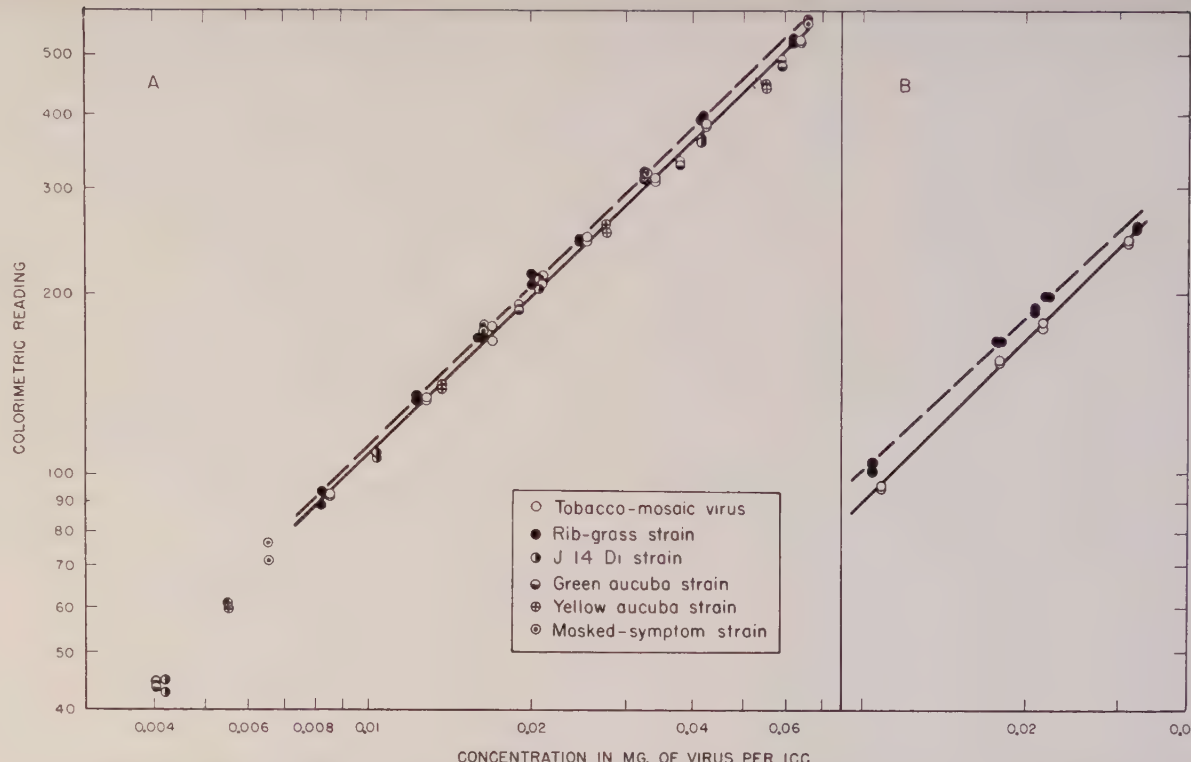

FIGURE 5. Relation between the logarithms of the concentrations and the logarithms of the colorimetric values produced by several strains of tobacco-mosaic virus with the Folin reagent in the presence of copper ions. A. Undiluted Folin reagent. B. Sixteen per cent Folin reagent. Solid lines are logarithmic regression lines calculated for the type strain, and dashed lines are logarithmic regression lines calculated for the rib-grass strain of tobacco-mosaic virus.

be significant for the existing degrees of freedom. It can be seen that the difference between the colorimetric values of the type strain and that of the rib-grass strain, as well as the differences between the values of the type strain and those of each of the other strains, are not significant. Evidently, the higher tyrosine content of 6.5 per cent in the rib-grass strain, as compared to 3.8 to 3.9 per cent tyrosine in the other strains of tobacco-mosaic virus, was not a strong enough factor to influence significantly the colorimetric values given by the rib-grass virus under the conditions of these experiments. The lower tryptophane content of 3.5 per cent in the rib-grass strain, as compared to 4.5 per cent in the type strain, evidently would tend to offset the slight increase in colorimetric values that might

TABLE II

RESULTS OF A STATISTICAL ANALYSIS OF THE DIFFERENCES BETWEEN THE COLORIMETRIC VALUES GIVEN IN THE PRESENCE OF COPPER IONS: BY THE TYPE STRAIN AND FIVE OTHER STRAINS OF TOBACCO-MOSAIC VIRUS WITH THE UNDILUTED FOLIN REAGENT, AND BY THE TYPE STRAIN AND THE RIB-GRASS STRAIN WITH THE DILUTED FOLIN REAGENT

Concn. of Folin reagent in per cent	Strains of tobacco-mosaic virus being compared	Values of t at different concentrations of the virus		Values of t required for the difference to be significant at $p = 0.01$
		0.03 mg./cc.	0.05 mg./cc.	
100	Type strain and Yellow aucuba	0.65	0.65	2.85
"	Type strain and Green aucuba	0.25	0.13	2.85
"	Type strain and Masked symptom	0.37	0.55	2.85
"	Type strain and J 14 D 1	0.06	0.92	2.85
"	Type strain and Rib-grass	1.22	1.25	2.76
		0.01 mg./cc.	0.02 mg./cc.	
16	Type strain and Rib-grass	3.87	3.19	2.98

be produced by the higher tyrosine content. By diluting the Folin reagent, it is possible to create conditions under which tryptophane contributes very little towards the colorimetric values given by a protein, the color now being chiefly attributable to its tyrosine content. Experiments performed under such conditions would be expected to result in a more pronounced difference between the colorimetric values produced by the rib-grass and the type strain of tobacco-mosaic virus. Colorimetric determinations of these two strains were repeated, using different dilutions of the Folin reagent. The results obtained with a 16 per cent Folin reagent, represented in Figure 5 B, indicate that higher colorimetric values were obtained for the rib-grass strain than for the type strain. From a statistical analysis of the data, the difference was found to be significant (Table II). These results confirmed the previously made assumption that by using a

dilute Folin reagent it is possible to differentiate between the rib-grass and type strain of tobacco-mosaic virus by means of the colorimetric method.

It has been observed in these experiments that in the presence of copper, two-thirds of the colorimetric value developed by tobacco-mosaic

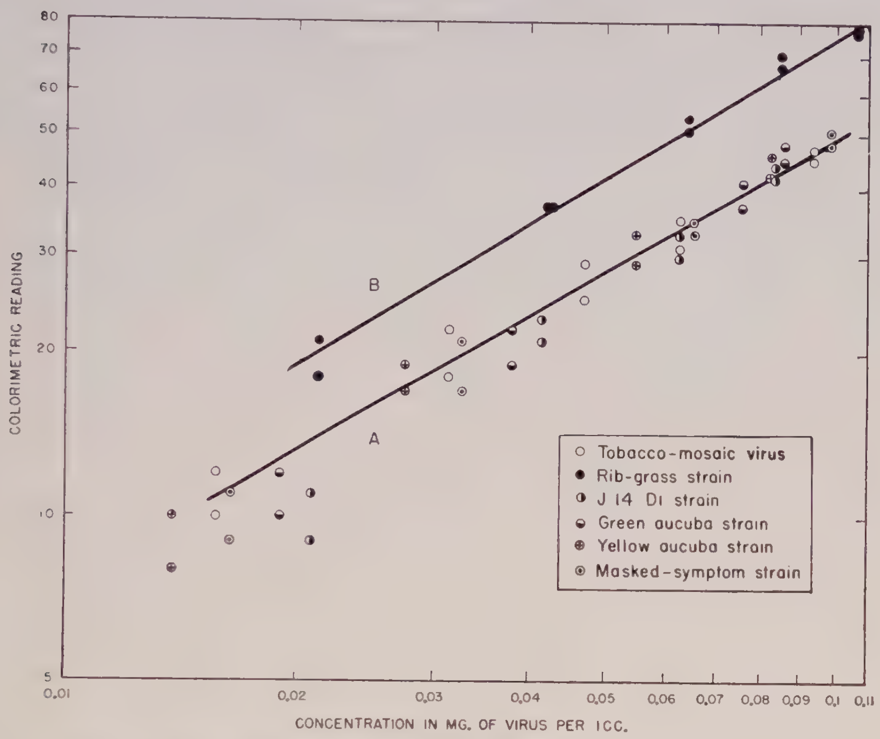

FIGURE 6. Relation between the logarithms of the concentrations and the logarithms of the colorimetric values produced by several strains of tobacco-mosaic virus with the Folin reagent in the absence of copper ions. Line A and line B are logarithmic regression lines calculated for the type strain and the rib-grass strain, respectively.

virus with the Folin reagent are due to some substances other than tryptophane and tyrosine, whereas in the absence of copper, these substances evidently do not contribute any colorimetric value to the reaction mixture. The difference between the colorimetric values of two strains differing in their tyrosine content would be expected to be more pronounced in the absence than in the presence of copper. The results of a series of colorimetric determinations of the six strains of tobacco-mosaic virus which were performed in the absence of copper (Fig. 6) indicate that much higher colorimetric values were obtained from the rib-grass than from any of the other five strains. Statistical analyses of the results (Table III) reveal that

the difference between the colorimeter scale readings of the rib-grass and the type strain are statistically significant at all three concentrations used for comparison. There is no significant difference between the colorimetric value of the type strain and the four strains of tobacco-mosaic virus which do not differ appreciably from the type strain in tyrosine content.

TABLE III

RESULTS OF A STATISTICAL ANALYSIS OF THE DIFFERENCES BETWEEN THE COLORIMETRIC VALUES GIVEN BY THE TYPE STRAIN AND FIVE OTHER STRAINS OF TOBACCO-MOSAIC VIRUS WITH THE FOLIN REAGENT IN THE ABSENCE OF COPPER IONS

Strains of tobacco-mosaic virus being compared	Values of t at different concentrations of the virus			Values of t required for the difference to be significant at $p = 0.01$
	0.03 mg./cc.	0.05 mg./cc.	0.08 mg./cc.	
Type strain and Yellow aucuba	0.110	0.201	0.512	2.97
" " " Green aucuba	0.202	0.140	0.008	2.97
" " " Masked symptom	0.624	0.265	0.095	2.97
" " " J 14 D 1	0.461	0.200	0.072	2.97
" " " Rib-grass	3.726	4.104	4.434	2.92

SUMMARY AND CONCLUSIONS

1. Quantitative micro-determinations of minute amounts of tobacco-mosaic virus can be performed with the aid of a Klett-Summerson photoelectric colorimeter using the Folin phenol reagent. The total weight of the virus required for the determinations falls within the range of 0.006 mg. to about 0.600 mg. virus.

2. The relation between the virus concentration and the colorimetric scale readings can not be expressed entirely satisfactorily by a single regression line, either arithmic or logarithmic, calculated for data covering the whole range of the concentrations used in the experiments. This relation can be best expressed for concentrations of about 0.008 to 0.060 mg. per cc. of reaction mixture by a logarithmic regression line calculated for this range, and for concentrations below 0.008 mg. per cc., either by a different logarithmic regression line or by an arithmic regression line fitting these lower concentrations.

3. The photoelectric colorimeter can be calibrated rapidly by determining in duplicate the colorimetric readings of only a few solutions of known concentrations, plotting the results on logarithmic paper, drawing two straight lines, one for each of the two ranges of concentration, and using these two logarithmic straight lines as calibration lines. Thus the customary long procedure of making calibration curves can be eliminated.

4. Tyrosine, tryptophane, and cysteine, the three amino acids generally considered responsible for the color developed by proteins with the

Folin reagent, in case of tobacco-mosaic virus, contribute not more than one-third of the colorimetric value given by this protein with the phenol reagent in the presence of copper. In the absence of more than a trace of copper ions the colorimetric values produced by tobacco-mosaic virus with the Folin reagent are much lower than the theoretical values calculated on the basis of the tyrosine, tryptophane, and cysteine content of the virus.

5. The colorimetric values obtained for the rib-grass strain are higher than those for the type strain of tobacco-mosaic virus. This difference is found to be statistically significant when the reaction is allowed to proceed in the presence of not more than mere traces of copper ions. The difference is also significant when too large an excess of the Folin reagent is avoided. The colorimetric method is thus suitable for the differentiation between two strains of tobacco-mosaic virus one of which is known to differ from the other in its tyrosine and tryptophane content.

6. There is no significant difference between the colorimetric values given with the Folin reagent by the type strain of tobacco-mosaic virus and four other strains of this virus which do not differ appreciably from the type strain in their tyrosine and tryptophane content.

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CARBON DIOXIDE STORAGE. XII. GERMINATION OF SEEDS IN THE PRESENCE OF CARBON DIOXIDE

Norwood C. Thornton

Previous investigations have shown that carbon dioxide may be used as a stimulant for the germination of lettuce (7) and cocklebur (6) seeds, while other workers (2, 3, 4) have reported its general inhibitory effect on the germination of seeds. Tests herein reported on the germination of various economic seeds show that most of those tested were able to initiate growth in the presence of exceedingly high concentrations of carbon dioxide.

MATERIAL AND METHODS

Seeds of buckwheat (*Fagopyrum esculentum* Moench.), cabbage (*Brassica oleracea* L. var. *capitata*), *Delphinium*, onion (*Allium cepa* L.), pepper (*Capsicum frutescens* L. [*C. annuum* L.]), pea (*Pisum sativum* L.), radish (*Raphanus sativus* L.), sunflower (*Helianthus annuus* L.), tomato (*Lycopersicon esculentum* Mill.), and wheat (*Triticum vulgare* L.) were obtained from seed houses in the winter of 1937 and germinated under the various conditions of these experiments. The germinating medium was moist cotton placed in either 500 or 1000 cc. Erlenmeyer flasks. The flasks were closed with corks and sealed with DeKhotinsky cement. Provision for placing the gas mixtures on the seeds was made by having a large bore glass tube passing through and sealed into the cork. This tubing was provided with a glass cap of larger bore than the tube which could be placed over the tube and sealed with a drop of warm cement or could be unsealed for changing of gas mixtures by the application of a minimum amount of heat.

The gas mixtures, containing 20 per cent of oxygen in nitrogen for control treatment or in combination with various percentages of carbon dioxide and nitrogen to make 100 per cent, were obtained from cylinders of the compressed gases. The procedure for mixing the gases was the same as already reported (5). The gas mixtures were placed in the flasks by having a small bore glass tube attached to the outlet of the mixing bottle and passed through the inlet tube to the bottom of the flask. Sufficient gas was passed through the flasks to give at least seven complete changes of the air and this was found by analyses to be ample to insure that the seeds were exposed to the desired mixture of gases.

The various temperatures were maintained by storing the flasks in electrically controlled ovens at the desired temperatures. The flasks were filled with the gas mixtures and then sealed while being cooled or heated to the desired temperature. The gas mixtures were also brought to the storage temperature by being passed through a coil immersed in a water bath. These precautions were taken to prevent undue pressure or vacuum in the flasks during germination at the various temperatures.

TABLE I

PER CENT GERMINATION OF SEEDS WHEN HELD AT VARIOUS TEMPERATURES IN DIFFERENT PERCENTAGES OF CARBON DIOXIDE*

Seed	Days for germ.	5° C.							10° C.							15° C.							20° C.						
		0	5	10	20	40	60	80	0	5	10	20	40	60	80	0	5	10	20	40	60	80	0	5	10	20	40	60	80
Buckwheat	7	0	0	0	0	0	0	0	72	56	36	0	0	0	0	96	96	100	88	20	0	0	100	88	96	92	56	0	0
Cabbage	13	24	32	16	8	8	8	4	48	64	28	28	12	12	8	32	36	40	24	24	20	20	44	48	40	48	40	32	20
Delphinium**	19	0	0	0	0	0	0	0	44	28	16	0	0	0	0	48	64	52	24	0	0	0	44	56	48	12	0	0	0
Onion	13	0	0	0	0	0	0	0	16	24	8	0	0	0	0	52	44	52	20	4	4	4	20	16	4	16	12	0	0
Pepper	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	60	56	40	4	0	0	0	60	76	68	48	8	0	0
Pea	12	50	25	70	25	0	0	0	65	60	70	40	15	0	0	75	95	100	100	70	15	10	85	80	70	85	70	30	15
Radish	12	0	0	0	0	0	0	0	92	56	20	12	0	0	0	96	100	80	60	20	0	0	100	92	96	100	76	56	32
Sunflower	11	65	55	65	5	0	0	0	95	100	100	85	25	0	0	80	90	70	90	85	65	30	40	70	80	65	50	80	50
Tomato	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	96	100	92	92	0	0	0	92	100	100	100	92	20	0
Wheat	7	100	96	100	20	0	0	0	100	100	96	100	64	0	0	100	100	100	100	100	100	20	100	100	100	100	92	96	88

TABLE I (*Continued*)

Seed	Days for germ.	25° C.							30° C.							35° C.						
		0	5	10	20	40	60	80	0	5	10	20	40	60	80	0	5	10	20	40	60	80
Buckwheat	7	100	96	96	96	52	32	8	88	88	92	84	72	20	0	76	96	76	84	80	52	8
Cabbage	13	36	56	48	52	44	28	12	24	20	24	20	40	16	16	28	28	24	28	20	28	12
Delphinium**	19	72	56	48	36	0	0	0	8	16	8	4	0	0	0	0	0	0	0	0	0	0
Onion	13	48	48	32	36	16	4	0	16	20	28	20	24	16	12	16	12	16	12	8	0	0
Pepper	15	60	68	68	56	16	0	0	64	64	64	36	20	8	0	0	16	20	4	0	0	0
Pea	12	85	100	95	85	85	65	40	85	90	75	70	65	30	20	—	—	—	—	—	—	—
Radish	12	84	92	88	92	92	64	48	92	84	84	80	80	64	52	60	52	44	48	28	12	0
Sunflower	11	45	50	65	45	70	35	35	45	45	70	35	55	45	30	40	50	45	40	30	25	30
Tomato	11	100	96	88	96	92	4	4	88	80	92	88	64	8	12	56	60	64	32	4	0	0
Wheat	7	100	100	100	100	96	100	84	100	100	100	100	92	92	64	96	100	92	92	92	72	20

* 20 per cent oxygen present in each treatment.
** Perennial.

The seeds were not pre-soaked but were placed on the moist cotton by means of a glass tube attached to a funnel after the cork had been sealed into the neck of the flask and just preceding the introduction of the gas mixture. The number of seeds (25 large or 50 small) used was necessarily small in order to facilitate counting of germinations during the tests.

EXPERIMENTAL RESULTS

As shown in Table I seeds vary in their ability to initiate germination in the presence of carbon dioxide. Wheat germinated in all concentrations of carbon dioxide at temperatures from 15° to 35° C. and was inhibited only by high concentrations of carbon dioxide at 5° and 10° C. On the other hand, delphinium seeds were inhibited by 20 per cent at 10° C. and by any concentrations of carbon dioxide above 20 per cent at 15° to 30° C. The other seeds that were tested showed responses to carbon dioxide treatment intermediate between these two extremes. Some seeds, e.g., the pepper and tomato, germinated best within a relatively narrow range of temperatures, 15° to 35° C., and were unlike cabbage, pea, sunflower, and wheat which germinated over the whole range of temperatures studied. In general, carbon dioxide was more effective in retarding germination at the lower temperatures, least effective at the intermediate, and again more effective at the highest temperature. Considerable variation was found with the different seeds tested. Tomato seeds were completely inhibited by 40 per cent carbon dioxide at 15° C., not affected by this concentration at 20° and 25° C., retarded at 30° C., and almost completely inhibited again at 35° C. Onion, pepper, and radish seeds show somewhat the same response as the tomato. Wheat seeds were inhibited by 40 per cent carbon dioxide at 5° C. and by 60 per cent at 10° C., but at all other temperatures it was only retarded in germination by 80 per cent of carbon dioxide. The most effective retardation in germination of wheat by 80 per cent carbon dioxide was obtained at 15° and 35° C. Some effect was observed at 30° C., and only a slight effect was found at 20° and 25° C. It is evident from the results given in Table I that the effectiveness of carbon dioxide in retarding germination of seeds varies not only with the species but also with the temperature at which they are held for germination.

Moist seeds usually show a retarded rate of germination as the concentration of carbon dioxide is increased. However, as shown in Table II, the retardation is only for a short period of time until the seeds become adjusted to the existing conditions. A comparison of the data in Table II with the final germination percentages given in Table I shows that the predominance of germination takes place in the first few days after the dry seeds are placed on the moist cotton. Thus it is indicated that these seeds are quite tolerant of carbon dioxide, and extremely high concentrations must be present to cause inhibition. If the seeds at first inhibited continue to be exposed to a concentration of carbon dioxide, they will become adjusted so that some germination may take place; thus is demonstrated

the ineffectiveness of carbon dioxide as a general inhibitor of germination of seeds.

The data in Table II indicate that cabbage may be stimulated to germinate by exposure to low concentrations of carbon dioxide as was found with lettuce (7).

After initiating germination in the various concentrations of carbon dioxide the roots and tops continued to grow. Measurements were made and the average growth of the seedlings of sunflower and wheat are recorded in Table III. In general, both the top and root growth of the seedlings exposed to carbon dioxide were retarded below that obtained in the absence of carbon dioxide. The length of growth of the seedling after germination is variable depending upon the seeds; some, like pea, cabbage, and especially lettuce (7), will do no more than initiate germination in carbon dioxide while others, such as tomato and those shown in Table III, will grow to considerable length. Seeds that start to germinate in carbon dioxide will continue growth when removed to air. Also, seeds that are inhibited from germinating by high concentrations of carbon dioxide will germinate without any indication of injury when the gas is removed.

DISCUSSION

Contrary to the conclusion of Kidd and West (4, p. 457) "that carbon dioxide in relatively small quantities in the atmosphere inhibits the germination of seeds," Kidd's (2) data and those reported in this paper show, in general, that high concentrations of carbon dioxide are necessary to inhibit germination of many seeds. Kidd's early data (2), much of which has been overlooked in reviews, show that barley, bean, cabbage, onion, and pea seeds were respectively inhibited only by 38, 45, 25, 30, and 50 per cent or higher concentrations of carbon dioxide at 17.5° or 20° C. Although there is no close agreement between these data and those given in this paper, the results do show that the general conclusions drawn from the work on white mustard (2, 3, 4) do not hold for all seeds. Kidd found white mustard seeds to respond quite readily to treatment with carbon dioxide, being inhibited by 20 per cent concentration at 17.5° C. or 3 to 4 per cent at 3° C. and readily developing a state of dormancy that could be eliminated only by drying the seeds or removing the seed coat. The response of white mustard is an exceptional case, and such results should not be made the general rule as to the effect of carbon dioxide on seed germination.

Limiting an experiment in regard to condition, material, or time may result in misleading conclusions. The effect of carbon dioxide on white mustard seed is to cause dormancy; on lettuce, to force germination under conditions where dormancy is produced; on wheat seeds, to permit germination even in very high concentrations of the gas; and on delphinium, to retard its germination. Thus the choice of only one seed would have misled one in forming general conclusions as to the effect of carbon dioxide on seed germination.

TABLE II

PERCENTAGE GERMINATION OF SEEDS AT VARIOUS INTERVALS DURING EXPOSURE TO CARBON DIOXIDE AT 25° C.

Seed	Days germ.	Per cent carbon dioxide in atmosphere						
		0	5	10	20	40	60	80
Buckwheat	1	88	80	60	40	0	0	0
	2	100	96	92	92	4	0	0
	3	100	96	96	92	28	12	4
Cabbage	1	8	32	0	12	4	8	4
	2	32	52	24	28	24	16	4
	4	36	56	48	44	32	28	12
	6	36	56	48	52	40	28	12
Pea	1	0	0	0	0	0	0	0
	3	50	40	30	20	25	10	0
	4	80	85	55	75	65	25	5
	5	85	90	95	80	85	40	25
Radish	1	4	0	0	0	0	0	0
	2	84	84	84	52	4	0	0
	3	84	92	88	88	32	12	0
	5	84	92	88	92	68	52	20
Sunflower	1	10	20	5	15	5	0	5
	2	45	45	55	30	30	10	5
	4	45	50	65	35	60	35	35
Tomato	2	20	8	16	4	0	0	0
	4	92	96	84	92	8	0	0
	6	96	96	88	96	40	0	0
Wheat	1	76	40	16	32	4	8	4
	2	100	100	100	100	52	8	12
	3	100	100	100	100	96	68	32

TABLE III

AVERAGE GROWTH IN CM. OF SUNFLOWER AND WHEAT SEEDLINGS AT THE END OF THE PERIOD OF EXPOSURE TO CARBON DIOXIDE AT VARIOUS TEMPERATURES

Seeds	Per cent CO_2	10° C.		15° C.		20° C.		25° C.		30° C.		35° C.	
		Top	Root	Top	Root	Top	Root	Top	Root	Top	Root	Top	Root
Sunflower	0	2.0	0.2	6.5	1.0	11.0	1.0	5.0	3.0	3.5	1.5	5.0	2.0
	5	2.0	0.2	8.0	3.0	4.0	1.0	5.0	3.0	3.5	2.0	2.0	2.0
	10	1.5	*	6.5	3.0	4.0	1.0	4.0	2.0	4.0	1.2	3.0	2.0
	20	0.1	*	2.0	1.0	6.0	3.0	5.5	2.0	4.0	1.5	4.0	3.0
	40	*	*	2.0	0.3	4.0	1.0	2.5	0.8	4.5	1.0	3.0	2.0
	60			1.5	*	2.0	*	1.5	*	2.0	*	3.0	0.2
	80			*	*	1.8	*	1.5	*	2.0	*	2.0	*
Wheat	0	1.0	1.2	8.5	2.0	15.0	2.0	10.0	3.0	10.0	2.0	4.0	1.5
	5	1.0	1.2	7.0	2.5	13.5	3.0	9.5	2.0	9.0	3.0	5.0	1.3
	10	0.8	0.5	8.0	1.5	10.0	2.0	7.5	2.0	7.0	2.0	4.0	1.0
	20	1.0	*	4.0	2.0	5.5	2.5	6.5	3.0	5.0	2.5	3.0	1.5
	40	0.7	*	0.8	1.0	2.3	2.0	3.0	3.5	2.0	2.2	2.0	1.0
	60			0.2	*	1.0	0.6	2.0	2.2	3.5	2.5	1.0	1.0
	80			0.2	*	0.6	*	0.7	0.4	0.6	*	0.5	*

* Growth could not be conveniently measured.

The results of these experiments show that for usual growing conditions one need not consider the effect of carbon dioxide produced in the soil on the germination of seeds. According to the work of Furr and Aldrich (1), however, one should consider the condition of the soil from the standpoint of supplying oxygen which may become a limiting factor in seedling growth more effective than an accumulation of carbon dioxide.

SUMMARY

Seeds of buckwheat, cabbage, delphinium, onion, pepper, pea, radish, sunflower, tomato, and wheat will germinate in much higher percentages of carbon dioxide than may be present under the average conditions of germination.

Tests made at seven temperatures ranging from 5° to 35° C. show that from 40 to more than 80 per cent of carbon dioxide is necessary when a normal supply of oxygen is present, to inhibit germination of the seeds except buckwheat, delphinium, and onion which were inhibited by 20 per cent only at 10° C.

Cabbage, pea, sunflower, and wheat initiated germination in all concentrations of carbon dioxide at all germinating temperatures except at 5° and 10° C. when high concentrations were effective in retarding germination of all but cabbage.

The inhibiting effect of carbon dioxide was greatest at the lower temperatures, least at intermediate, and again somewhat effective at the highest temperature tested.

The delaying action of carbon dioxide on the germination of seeds and seedling growth is shown in the tables of data.

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DORMANCY, BUD GROWTH, AND APICAL DOMINANCE REGULATED BY OXYGEN IN FRESHLY-HARVESTED POTATO TUBERS

NORWOOD C. THORNTON

The failure of bud growth on freshly-harvested potatoes is due to the ready permeability of the tissue to oxygen and to the oxygen content of the air being too high (6). Growth of such buds can be initiated at once if the oxygen concentration of the surrounding air is reduced from 20 per cent to approximately 2 to 10 per cent. As the potato tuber ages, its permeability decreases with time by the development of a thickened periderm until a point is reached at which the supply of oxygen to the bud tissue has been sufficiently reduced to furnish these low concentrations which are required for sprouting to proceed. Besides favoring the early growth of dormant buds, low concentrations of oxygen cause the elimination of dominance of the apical bud over the lateral buds of each eye and of the buds in the eyes at the apices of the tuber over those near the basal or stem end of the tuber.

Apical dominance in the potato has been a subject of investigation for many years. Appleman (1) demonstrated how apical dominance could be overcome by cutting the tubers in order that there would be no connection between the respective eyes. Denny (3) found thiourea in 3 per cent aqueous solution extremely effective in promoting the growth of multiple sprouts from each eye of the freshly-harvested potato, thus overcoming not only apical dominance of the seed end over the stem end but also of one bud over all other buds in each eye. Denny (4) reported that ethylene chlorohydrin was also effective in promoting multiple sprouting of buds of potato tubers. Bushnell (2) and Werner (7) have reported normal multiple sprout development of potatoes many months after they had completed their dormant period. They found that immediately after the completion of the dormant period the seed pieces produced only one sprout per eye, but with succeeding plantings at monthly intervals thereafter there was a progressive increase in the number of sprouts produced from each eye. Appleman (1) reported a similar condition existing over a period of 12 months with whole tubers following repeated removal of sprouts as they were produced.

MATERIAL AND METHODS

Potato (*Solanum tuberosum* L.) tubers of the varieties Irish Cobbler, from lots harvested either in North Carolina, New Jersey, or the Institute gardens, and Bliss Triumph, harvested from the Institute gardens, were

used in these tests. The potatoes were subjected to treatments immediately upon being harvested and after being held for not more than ten days after harvesting. Both whole tubers and tubers cut into one-eye pieces were used. Moist and relatively dry conditions were maintained during the period of treatment, which was continued for 21 to 24 days in some tests. The tubers in the containers were either covered with moist sphagnum moss or held dry yet exposed to a high relative humidity. Also, potatoes were held in an open room at 23° to 28° C. where they were exposed to approximately 50 per cent relative humidity. All tests were carried out at room temperature which varied from 23° to 28° C. during the spring, summer, and autumn months.

The potatoes were treated at atmospheric pressure with pure oxygen and nitrogen and with gas mixtures consisting of various percentages by volume of oxygen and nitrogen (6). Carbon dioxide was continuously removed from around the tubers during treatment in order that there would be no stimulation of bud growth from this source (5). Gas analyses of the storage atmosphere were made at frequent intervals to insure the absence of carbon dioxide and the presence of the desired concentration of oxygen. To obviate any considerable change in the oxygen and carbon dioxide content of the container during a 24-hour period, it was necessary to have the volume of the container large in proportion to the number of tubers. For this reason the number of tubers in each test had to be limited to four or six per 8-liter tin container.

Observations were made not only on the production of visible sprouts at the eyes of the intact tuber during treatment, but also on the ability of one-eye pieces of treated tubers to produce sprouts in soil in a basement room at 23° to 28° C., also on the growth of plants in soil in the greenhouse, and in the open field at the usual summer temperatures.

RESULTS

The sprouting of dormant potatoes within seven to nine days after harvest has been obtained by treatment with reduced concentrations of oxygen; 2 per cent with dry- and 5 to 10 per cent with moist-held potatoes. If the potatoes were allowed to dry for as much as ten days following harvest and before placing under treatment, sprouting was observed 12 days after starting the treatment. Storage of dormant potatoes in pure nitrogen also brought about the growth of the buds, but at a slower rate and following a longer period of treatment than when a small amount of oxygen was present. Treatment with pure nitrogen cannot be carried on without various precautions such as thorough drying of potatoes following harvest and daily changing of gas as has been discussed in a previous report (6).

The exposure of freshly-harvested potato tubers to reduced oxygen concentrations initiated more changes in the tuber than breaking the dor-

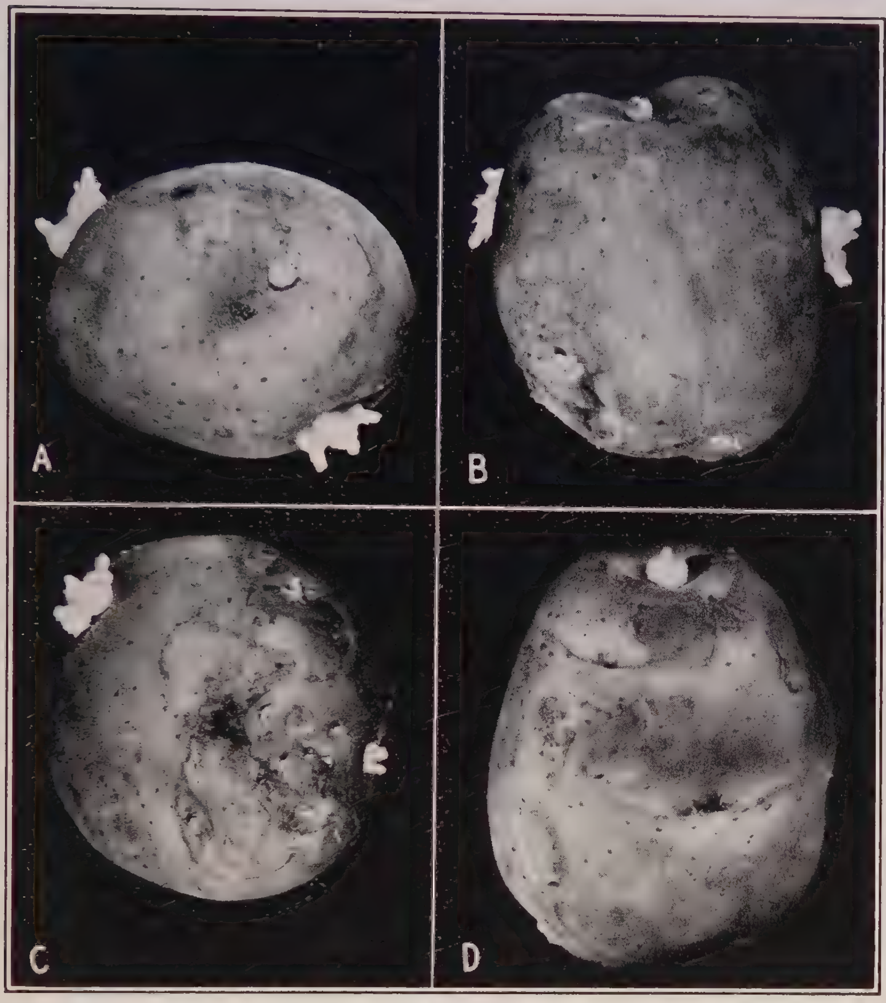

FIGURE 1. Apical dominance of buds of Irish Cobbler potatoes was overcome by treatment with 0 to 5 per cent of oxygen in dry and 0 to 10 per cent of oxygen in moist condition at 23° to 28° C. (A) Stem end view, (B) side view, and (C) apex end view. (Photographed after 24 days of treatment following harvest.) (D) Apical dominance of bud in eye at apex end of untreated potato at completion of its usual period of dormancy.

mancy. This treatment has completely eliminated apical dominance in the potato. As shown in Figure 1 A, sprouts were produced in the eyes at the basal end of the tuber adjacent to the stem scar tissue. A side view of the tubers in Figure 1 B shows sprouts being produced not only near the basal end but in the eyes spread over the entire surface of the tuber, irrespective of their position in relation to the apex of the potato tuber. A view of the apical end of the tuber, Figure 1 C, shows large buds and many small buds

growing in each and every eye of the tuber. When the buds start to grow while in the treatment they appear in numbers from almost every eye at the same time irrespective of the position of the eye in relation to the apex of the tuber. All of the buds continued to grow during the 21 to 24 days of treatment but some developed at a more rapid rate than others as shown by the differences in size of the buds on the tubers in Figure 1 A, B, and C. Usually, but not always, the fastest growing buds in the early period of treatment were in the eyes located just below the groups of eyes at the apical end of the tuber. As the period of treatment was extended, buds in other eyes also developed at a more rapid rate. In addition to the loss of apical dominance in the whole tuber, the treatments with reduced oxygen destroyed the apical dominance of one bud over all lateral buds of the individual eyes of the potato tuber (Fig. 1 A, B, and C). Irish Cobbler potatoes produced three or, most often, four sprouts per eye while Bliss Triumph potatoes produced from three to five sprouts per eye during exposure to low concentrations of oxygen in either a moist or a relatively dry condition.

Figure 1 D shows the usual procedure of sprouting of potato tubers, after the normal completion of dormancy; apical dominance is demonstrated with the rapid growth of one bud per eye in the center or topmost eye of the apical end of the potato tuber. This is the usual type of sprout production of tubers held in 20 to 100 per cent of oxygen. When the tuber has completed its dormancy and is cut into one-eye pieces, each eye is free of the controlling influence of the apical eye and sprouts readily irrespective of its former position in the tuber. However, there is another demonstration of apical dominance in the non-dormant potato tuber as shown by the growth-retarding influence of the topmost bud of the eye over all lateral buds of that eye. Thus in most cases the one-eye piece of tuber will produce only one shoot, just after completing the dormant period, and it will grow to maturity unless it is damaged, in which case another bud is then released to produce the plant.

This elimination of apical dominance takes place not only in the whole tuber or in one-eye pieces of tubers during the period that they are in the low oxygen treatment, but it carries over into the production of more than one plant per one-eye piece of tuber when planted in soil. When 24 one-eye pieces from each treatment of whole tubers were planted in soil, these developed an average of 1.7 plants from each piece of tuber treated with 2 per cent of oxygen, 1.3 plants from treatment with pure nitrogen, and 1.0 plants from treatments of 20 per cent or higher percentages of oxygen. These data, shown in Table I, were taken from counts of the plants grown from one-eye pieces of treated tubers and the data were recorded 38, 58, and 87 days from harvest which included the 24 days the whole tubers were exposed to various concentrations of oxygen.

Usually intact potato tubers exposed to low concentrations of oxygen produced multiple sprouts in all eyes of the tuber at about the same time. As the treatment is continued the buds in some eyes will grow at a more rapid rate, thus producing the condition shown in Figure 1 where there are large, medium, and small buds on the same tuber. If the tubers are cut into one-eye pieces and planted in soil, various numbers of plants are produced depending apparently upon the size of the bud development at time of planting. One-eye pieces having large buds as shown in Figure 1 produced three or four plants of equal vigor; medium size buds produced two vigorous plants and either one or two small plants per eye; small buds produced one vigorous plant and either a weak plant and an enlarged bud or two or more buds that developed about one-half inch shoots. The most effective treatment containing low percentages of oxygen caused the development of enlarged buds in numerous eyes of the tubers as contrasted to enlarged buds in only a few eyes in the case of treatment with pure nitrogen.

TABLE I

AVERAGE NUMBER OF STEMS GROWING IN SOIL PER ONE-EYE PIECE CUT FROM TUBERS THAT WERE HELD RELATIVELY DRY IN VARIOUS PERCENTAGES OF OXYGEN FOR 24 DAYS

Treatment, percentages of		Stems per one-eye piece, Days after harvest		
O_2	N_2	38	58	87
0	100	1.3	1.3	1.3
2	98	1.5	1.7	1.7
5	95	1.3	1.6	1.6
10	90	1.0	1.2	1.2
20	80	1.0	1.0	1.0
40	60	—	1.6	1.0
80	20	—	1.0	1.0
100	0	—	1.0	1.0
Dry, in room		—	1.0	1.0

DISCUSSION

Reduced percentages of oxygen cause the early elimination of apical dormancy in freshly-harvested potato tubers which leads to the production of multiple plants from one-eye pieces. These results may be compared with the demonstration of apical dormancy by the development of one bud per tuber or one plant from a one-eye piece many weeks after harvest when the tuber has completed its dormant period. Reports (1, 2, 7) in the literature show that the tuber or one-eye piece will produce multiple sprouts after many months of storage following harvest and after the tuber has completed its period of dormancy. In the light of a study of the development of the periderm (6) during aging in storage it is possible that the tubers essentially develop a thickened layer that retards the passage of oxygen to

the bud tissue, thus bringing about the same result of multiple sprouting that reducing the oxygen concentration will do at the time the tubers are freshly-harvested and in their period of dormancy.

SUMMARY

The dormancy of freshly-harvested Irish Cobbler and Bliss Triumph potatoes was broken and bud growth was obtained within seven to nine days by continuous treatment of the tubers with 2 per cent of oxygen in relatively dry condition or 5 to 10 per cent of oxygen in moist condition at 23° to 28° C. Bud growth was produced on tubers stored in pure nitrogen for 18 days following harvest.

These treatments brought about the elimination of apical dominance of the buds in the apex eyes of the tuber over those in eyes at the basal or stem end. Also the treatments removed the controlling effect of the apical bud over the lateral buds of each eye so that many buds developed in each and every eye of the freshly-harvested potato.

Potatoes removed from the treatments were cut into one-eye pieces and planted in soil with the result that vigorous multiple plants were produced from the one-eye pieces that had been exposed to reduced percentages of oxygen.

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EVALUATING FUNGICIDES BY MEANS OF GREENHOUSE SNAPDRAGON RUST[1]

S. E. A. McCallan

A greenhouse method of evaluating fungicides by means of foliage diseases on potted tomato plants has been described in detail in a previous paper (6). Due to the specificity of many potential fungicides, especially among organic compounds, it is desirable to have methods representing the major groups of plant disease fungi. In the above case the diseases employed were Early Blight (*Alternaria solani*), Septoria Leaf Spot (*Septoria lycopersici*), and Late Blight (*Phytophthora infestans*). Another of the important groups of plant diseases is the Rusts. In selecting a suitable rust disease, the requirements set forth in the above paper were followed excepting that it appeared impossible to obtain a satisfactory major economic disease on a quick growing plant. Preliminary tests with Bean Rust and Stem Rust of Wheat were abandoned in favor of Snapdragon Rust. Apple Rust was not considered because of the impracticability of obtaining large numbers of plants.

Snapdragon or antirrhinum plants (*Antirrhinum niger* L.) are easily though slowly grown in the greenhouse and handle well when being sprayed. The rust organism, *Puccinia antirrhini* Diet. & Holw., is easily propagated in the greenhouse and will not spread readily to healthy plants if the latter are kept in a separate house. The disease lesions or rust pustules are easily identified and counted.

METHODS

The snapdragon seed was first sown in small flats, then later transplanted to large flats, and finally to four-inch pots. The plants were pinched back after the first transplants became established, and were trained to two stems. About four months are required to raise plants to a size suitable for use.

The apparatus and general technique employed with the tomato foliage diseases (6) have been followed throughout for Snapdragon Rust. The plants are placed on the turntable and sprayed for 30 seconds, using the nozzle of the paint gun adjusted to a vertical ellipse because of the greater height of the snapdragon plants. After drying, the plants are inoculated with a spore suspension by means of the hand atomizer and immediately placed in the high humidity infection chambers, where they remain for 24 hours and after which are returned to the greenhouse to await development of rust pustules. Since the rust, *Puccinia antirrhini*, is an obligate

[1] A preliminary report on this paper was presented before the American Phytopathological Society, Cincinnati, Ohio, December, 1944 (5).

parasite, spores are of necessity obtained from infected plants. The unsprayed but inoculated check plants from previous tests usually served as the means of culturing the fungus.

FACTORS INFLUENCING THE DISEASE IN CONTROL PLANTS

The various factors held to be of importance in the development of the disease on unsprayed plants have been studied.

VARIETIES

It is well known that snapdragon varieties differ markedly in their susceptibility to rust as well as in their growth habit. A comparison was made of 20 different varieties obtained from three seedsmen and planted on two different dates several months apart. The plants were inoculated with the rust in four different experiments and observations were also made on a number of growth habits.

The ideal snapdragon plant for spraying and evaluating fungicides should be moderately rust susceptible, of upright and robust growth habit. It should produce few or no axillary shoots and should have long internodes. The growth rate of the stems should be rapid and uniform, and the leaves should be large, broad, and uniform in size. Also because of visibility of pustules, the green-leaved varieties are preferable to the purple-leaved ones. Finally, well known standard varieties are the most desirable. The varieties were rated in these various categories and the results are given in Table I. Due to an error in ordering, six of the varieties obtained were "rust resistant" instead of being rust susceptible; however, all were not truly rust resistant as may be seen in Table I. On the basis of these results, it was decided to adopt Cheviot Maid Supreme as the standard variety although admittedly it is not ideal in all respects, particularly with reference to uniformity of stems and leaves. It appears impossible to obtain snapdrgon plants with the degree of uniformity possessed by tomato plants (6).

LEAF POSITION AND SIZE OF LEAVES

The effect of leaf position and size of leaves on the number of rust pustules was determined in a number of tests with the variety Cheviot Maid Supreme. The leaves were numbered serially, beginning with the bottom or oldest leaves. The total number of pustules appearing on both opposite leaves and both stems, i.e., four leaves were counted. Leaf areas were estimated by comparison with a chart on which were drawn a series of graded leaves ranging from 1 sq. cm. to 12 sq. cm., each succeeding leaf being approximately 1.2 times the area of the previous one. Typical results based on the mean of two tests totalling ten 12-inch tall plants are

shown in Figure 1. It will be noted that in this case the largest leaves occurred in the 9th position and that in general the largest leaves had the greatest number of pustules. The number of pustules per unit area is fairly constant over the middle range but falls sharply at the ends. The decrease

TABLE I

Classification of Snapdragon Varieties Regarding Desirability as Test Plants

Variety	Rust susceptibility	Erect and robust	Absence of axil. shoots	Long internodes	Stems		Leaves			Availability
					Size	Uniformity	Size	Uniformity	Greenness	
St. George	3	3	1	1	2	2	3	2	3	1
Amber Queen	3	1	1	1	1	3	1	2	3	0
Black Prince	2	3	0	1	2	3	1	3	0	0
Flame	3	1	1	0	0	3	2	3	3	0
Golden Queen	3	1	1	2	3	2	2	2	3	0
Nelrose	3	2	2	2	3	3	2	3	3	0
Othello	3	3	3	3	3	2	2	3	0	0
Silver Pink	2	3	0	1	2	3	1	3	3	0
Bertha Bauer	3	3	3	3	3	1	2	3	3	1
Cheviot Maid Supreme	3	3	2	2	2	2	2	2	3	3
Kirkwood Beauty	3	3	2	2	3	2	2	2	3	1
Yellow Perfection	2	2	1	2	2	2	1	2	3	0
Lucky Strike	2	1	2	2	2	2	2	2	3	3
Velvet Beauty	3	1	1	2	2	2	2	1	3	2
Alaska	1	1	1	3	3	3	2	1	3	2
Campfire	1	3	2	2	3	2	3	2	3	0
Copper Queen	0	2	3	3	3	2	3	2	3	2
Crimson	1	2	3	2	2	2	3	3	0	1
Loveliness	0	2	2	3	3	2	2	2	3	2
Yellow Giant	1	1	3	3	3	2	3	3	3	2

3 = good, 2 = fair, 1 = poor, 0 = very poor. In case of rust susceptibility, 3 = high, 0 = no.

in leaf size from No. 3 to No. 5 leaf is believed to be due to the setback following transplanting from flat to individual pots. Individual counts on each of the two alternate leaves as well as on the comparable leaves of each

TABLE II

Relation of Age and Size of Plant to Number of Rust Pustules

Age in weeks	Mean height in inches	Mean number of pustules	
		Per 20 leaves	Per 10 sq. cm.
13	5	387	259
14	9	675	465
16	13	816	445
19	24	656	327

paired stem in general agreed so closely that little was to be gained by making separate counts on each of the four leaves. It was thus decided to count the total pustules per plant appearing on the five adjacent sets of

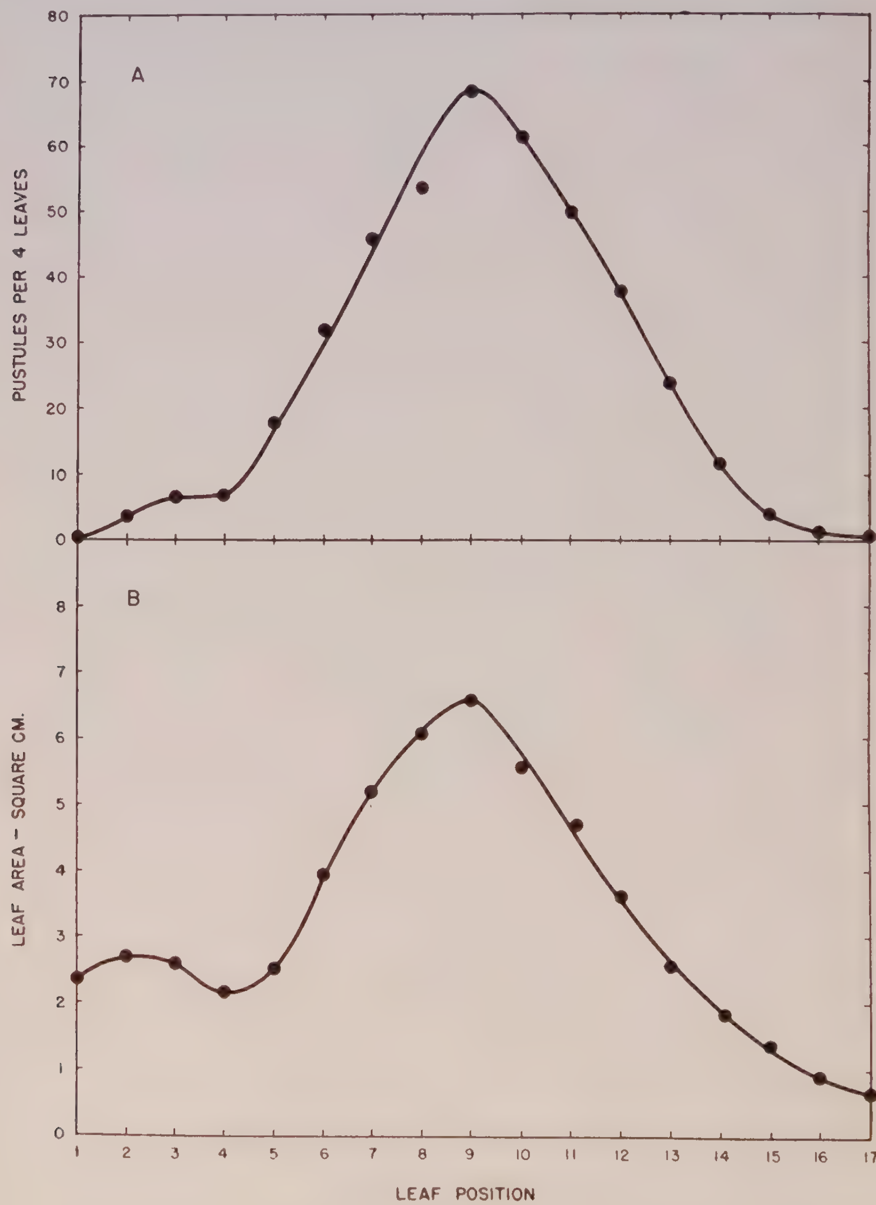

FIGURE 1. Relation of snapdragon leaf position to (A) number of rust pustules per four leaves and (B) leaf area per one leaf and one surface.

leaves having the greatest total number of pustules. In the case of Figure 1 A, this would be leaves Nos. 7, 8, 9, 10, and 11; the total number of leaves being 5×4, or 20. The results of further comparisons relative to counting pustules on a per leaf basis or a per unit area basis will be given later.

SIZE AND AGE OF PLANTS

A comparison was made of the effect of size and age of plants on the number of pustules per 20 leaves. Plants of four different ages were tested,

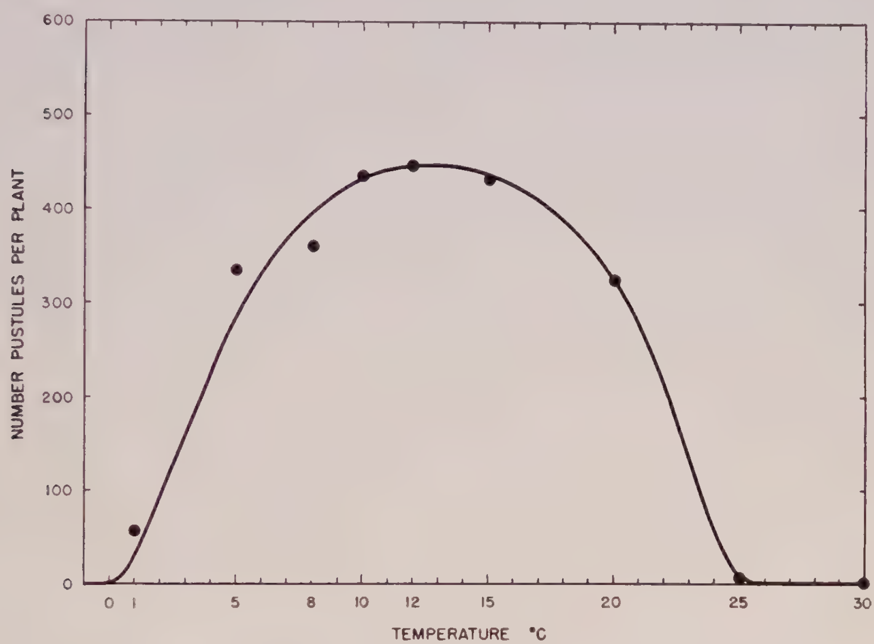

FIGURE 2. Effect of incubation temperature on infectivity of *Puccinia antirrhini* uredospores as measured by number of rust pustules per plant (20 leaves).

the differences in size were compensated for when inoculating, by having the total number of spores applied approximately proportional to the total leaf area of the plants. The data are given in Table II where it may be seen that the number of pustules or susceptibility decreases with extremes of age or size. Since the plants will grow at different rates, they are identified by size rather than age, and a height of about 12 inches is considered most satisfactory for the variety used.

TIME OF APPEARANCE OF PUSTULES

The first symptoms of the rust are seven to eight days after inoculation when small white swellings appear on the lower surface of the leaves. By

the tenth day about 50 per cent of the white swellings have ruptured, exposing the familiar brown uredinia. It has been observed over a period of several years that this stage is consistently attained on the 10th day and this is considered the ideal time for counting the rust pustules. By the 14th day secondary pustules have commenced to appear around the primary sorus and it is no longer possible to identify easily the original points of infection. Accordingly, all results have been taken on the 10th day after inoculation. Since the rust pustules can be identified so readily, it has not been considered necessary to correct for the variation of individual operator's counts as is required for the tomato diseases (6).

TEMPERATURE

Doran (2) has presented an astonishingly sharp temperature curve for the germination of uredospores of *P. antirrhini* on glass. The optimum was at 10° C. and if the temperature varied only 1° either way the germination dropped to one-third the value. Plants inoculated at 10° C. by Doran averaged 224 sori as compared to 12 for 15° C. and 6 for 18° C. A further study of temperature has been made, plants being inoculated at a series of nine different temperatures ranging from 1° to 30° C. The tests were performed at four different times with three plants for each test at each temperature. The various tests agreed within the experimental error and the mean results are shown in Figure 2. It will be seen that the temperature curve is relatively flat-topped, there being little difference within the range 5° to 20°, and no significant difference whatsoever within the range 10° to 15° C. These results are in accord with spore germination tests on glass performed at this time and also earlier (9, p. 19). Humidity infection chambers run at 10° to 15° C. would be most desirable for maximum infection of snapdragon plants. However, satisfactory infection has always occurred when the temperature of the room was not allowed to rise above 20° C.

SPORE CONCENTRATION

Several tests were performed in which the spore concentration of the inoculum was varied. It was found as in the tomato diseases, that the number of lesions or pustules was directly proportional to the number of spores. Under standard conditions, i.e., Cheviot Maid Supreme variety, plants 12 inches tall and temperature from 10° to 20° C., approximately 300 pustules may be expected on 20 leaves when a concentration of 100,000 spores per cc. is applied.

SPORE AGE

The viability of the uredospores diminishes as the leaves age or dry out. Doran (2) found that spores from excised shoots germinated little or none after six weeks regardless of temperature or humidity. A comparison

has been made of the germination of spores from sori on old leaves of mature pot-bound plants and spores from green leaves of young active plants. The former gave 41 per cent germination and the latter 65 per cent. It has not proved practical nor does it seem necessary to maintain a stock of plants of a constant age and constant elapsed time of infection to provide spores. Spores suitable for inoculation have always been obtained from the control plants of preceding tests.

VARIATION IN REPLICATE CONTROL PLANTS

It was observed in the studies with the tomato diseases (6) that the replicate control plants varied appreciably; the more in some diseases than in others. In the case of snapdragon plants the counts are taken on the paired stems and as shown before may be based on 20 leaves or on a given area of leaves. Available data were examined to determine the extent to which these factors contributed to the replicate plant variation. In order to have data of comparable magnitude the number of pustules per stem of ten leaves was converted to logarithms and an analysis of variance performed on replicate stems, i.e., paired stems, and on the corresponding replicate plants with results as follows:

	Degrees of freedom	Variance
Replicate stems	101	0.0124
Replicate plants	53	0.0232**

It will be seen that the variation of the paired stems is significantly less than that of the replicate plants. Thus the replicate stems contribute but little to the variation of the replicate plants and there is no reason for recording data separately for the individual stems.

The results of a comparison of recording data, on the same plants, on the basis of 20 leaves and on a basis of per unit area of 10 sq. cm. on replicate plant variance are given in Table III. As above, the number of pus-

TABLE III

Comparison of Replicate Plant Variances for Varieties and Methods of Recording Data. Logarithms of Pustules

Variety	D. F.	Per 20 leaves	Per 10 sq. cm.
Cheviot Maid Supreme	50	0.0253	0.0274
11 other varieties	99	0.0251	0.0279

tules were converted to logarithms. The comparisons were made both on the variety Cheviot Maid Supreme and on 11 other varieties mentioned in Table I. It will be noted that there was no significant difference between the two methods of recording data, nor between the variety Cheviot Maid

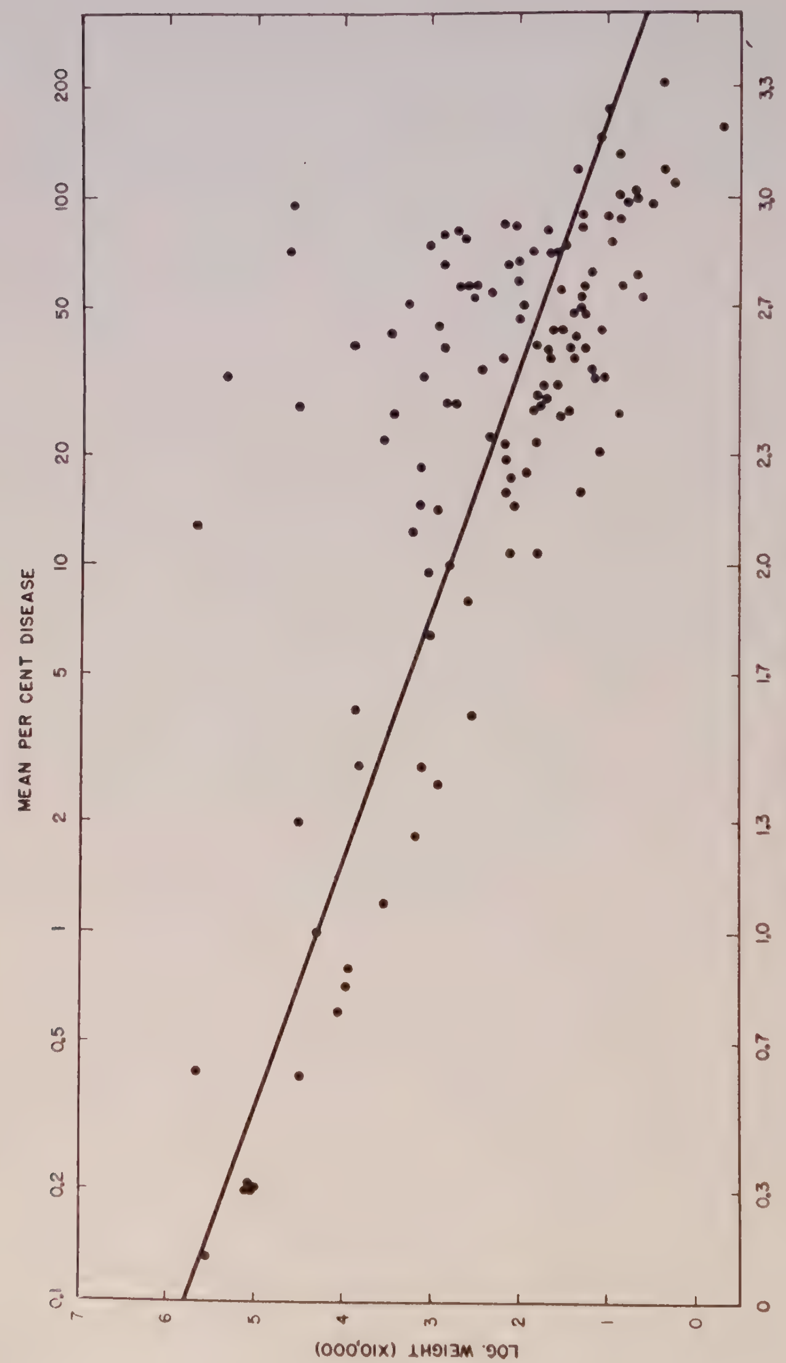

FIGURE 3. Regression of logarithm of weights on logarithm of mean per cent disease for Snapdragon Rust. Weights or reciprocal of variance derived from per cent disease on two replicate plants. $Y = 5.7786 - 1.4884\ X$.

Supreme and the mean of 11 other varieties. Individual comparisons of each of the 11 varieties (9 DF) with Cheviot Maid Supreme (50 DF) showed none significantly less. Hence the replicate plants of Cheviot Maid Supreme are in as close agreement or better than any of the other varieties and thus its selection as the standard variety is further justified. Since the laborious method of recording numbers of pustules on a unit area basis can not be shown to reduce the variation of replicate plants, the simpler method of recording on the basis of 20 leaves per plant will be continued as standard.

A comparison of the control replicate plant variance of Snapdragon Rust, namely 0.0232, with that obtained for the three tomato diseases (Table V, column 3) showed that the replicates did not agree as closely as those of Late Blight, did not differ significantly from Early Blight, and agreed more closely than those of Septoria Leaf Spot.

FUNGICIDE TESTS

Employing the standard conditions outlined above, fungicide tests were conducted in order to obtain basic data relative to the precision at different levels of control, reproducibility of tests, number of plants per dose, the slope of the dosage-response curve, and the degree of control attained by different types of fungicides. The same nine fungicides used in the tomato tests and previously described (6, footnotes 9 to 16) were employed, namely Bordeaux Mixture, Yellow Cuprocide, and Copper Fungicide "34" representing copper fungicides, Lime Sulphur, Mike Sulphur, and Dry Wettable Flotation Sulphur typifying sulphur fungicides, and Spergon,[2] Fermate,[3] and Thiosan[4] as examples of organic fungicides. Two plants were sprayed at each dosage, the dose ratio was 5 except for Spergon where it was $\sqrt{5}$, and four control plants were used. Each fungicide was tested at two different times and all three fungicides of the same group, e.g., the sulphur fungicides, were always run at the same time.

Weighting coefficients. When the per cent disease control was plotted against the dosage on logarithmic probability paper for the individual plants and tests, it was observed that the points could be fitted by a straight line and that there was less variation between duplicates at the higher per cent control values than at the lower. This is in general agreement with the results obtained with the tomato diseases. Employing the methods used previously (4) the empirical probit weights for the dosage response curves were obtained.

The logarithm of the weight, that is, the reciprocal of the logarithm of the variance, for the per cent disease of the two replicate plants was plotted

[2] Tetrachloro-para-benzoquinone 50%.

[3] Ferric dimethyldithiocarbamate 70%.

[4] Tetramethylthiuramdisulphide 50%.

against the logarithm of the mean per cent disease. This is illustrated in Figure 3 for the 126 pairs of replicate plant data available. The regression equation was determined to be $Y=5.7786-1.4884\ X$ and the standard error of estimate $S_{y\cdot x}$ 0.865. The standard error of the regression coefficient S_b was 0.117 which gives a highly significant t value of 12.7. When this regression coefficient was compared with that obtained for the tomato diseases no significant difference could be shown between the two ($t=0.427$). Thus it appears that the same natural laws govern the distribution of Snapdragon Rust pustules as do the distribution of Early and Late Blight lesions on tomato. Hence the probit weighting coefficients obtained for Early and Late Blight may also be employed for Snapdragon Rust.

A comparison was made between Snapdragon Rust and Early and Late Blight at the LD95 level. No significant difference could be shown between the weights of Snapdragon Rust and Early Blight, but between Snapdragon Rust and Late Blight a significant difference could be shown indicating that the weight of Late Blight was approximately four times that of Snapdragon Rust. This is in agreement with the results obtained above on replicate control plants.

Replicate test and replicate plant variances at LD95. Individual dosage response curves were plotted as probits against logarithm of dose for the replicate plants sprayed with the sulphur and organic fungicides and the dosage at probit 6.64, i.e., LD95, determined. The logarithmic dosages so obtained were submitted to an analysis of variance with results as shown

TABLE IV

ANALYSIS OF VARIANCE OF LOGARITHMS OF LD95 VALUES FOR SULPHUR AND ORGANIC FUNGICIDES AGAINST SNAPDRAGON RUST. POOLED RESULTS

	D. F.	Variance	Significance
Fungicides	4	1.4517	High/Fungicide×Tests
Tests	2	0.3141	High/Fungicide×Tests or Repl. plants
Fungicides×Tests	4	0.0242	No/Replicate plants
Replicate plants	12	0.0230	

in Table IV. It will be noted that there is a very marked test, that is, day-to-day effect, as is usually the case for greenhouse and laboratory tests of fungicides (7). The replicate plant variance is low as compared to that of tests and is approximately the same as the fungicide test interaction. Evidently little is to be gained by carrying replicate plants as was concluded for the tomato diseases, since they contributed but little to total variance and would not ordinarily be used for error.

A summary of comparative variance obtained for the three tomato diseases (6) and for Snapdragon Rust is given in Table V. The data on LD95 value from replicate plants are insufficient to show any significant

difference (column 5). However, the basic error term of fungicide × tests for Snapdragon Rust is significantly lower (last column), though the degrees of freedom available for the latter are few.

Dosage-response curves and relative control. The mean probit response at each dosage for all replicates was obtained and the dosage-response curves

TABLE V

Comparative Variances of Four Greenhouse Diseases

Disease	Log. lesions on replicate check plants		Log. LD95 values			
			Replicate plants		Fungicide × Tests	
	D. F.	V.	D. F.	V.	D. F.	V.
Tomato Late Blight	72	0.0091	18	0.011	321	0.1170
Tomato Early Blight	68	0.0159	18	0.046		
Tomato Septoria Blight	10	0.0886				
Snapdragon Rust	53	0.0232	12	0.0239	4	0.0242

plotted as shown in Figure 4 on a logarithmic probability scale. It will be noted that the organic fungicides have relatively steep slopes and were the most effective in disease control. The sulphur fungicides also with fairly steep dosage-response curves gave intermediate control, but the copper fungicides were wholly ineffective in control and possessed very flat curves. This trend associating steep dosage-response curves with effective control and flat curves with poor control or high LD values has been noted before in laboratory tests of copper fungicides and soluble heavy metals (8).

Fermate gave the best control of Snapdragon Rust. It has been shown by Hamilton *et al.* (3) that with another rust, Cedar Apple Rust, Fermate is remarkably effective under field conditions. It, of course, is generally recognized that the sulphur fungicides can be used effectively against rust

TABLE VI

Effectiveness of Different Fungicides Against Snapdragon Rust

Fungicide	LD95 in %*	Per cent disease at 0.2% spray conc.*
Fermate	0.0007	<0.1
Thiosan	0.0011	<0.1
Spergon	0.0086	<0.1
Lime Sulphur	0.022	<0.1
Flotation Sulphur	0.038	0.2
Mike Sulphur	0.230	7.0
Bordeaux Mixture	>5	54
Tennessee Cu "34"	>5	57
Yellow Cuprocide	>5	58

* Per cent total solids, except Bordeaux = % copper sulphate and lime sulphur = % 32° Baumé stock.

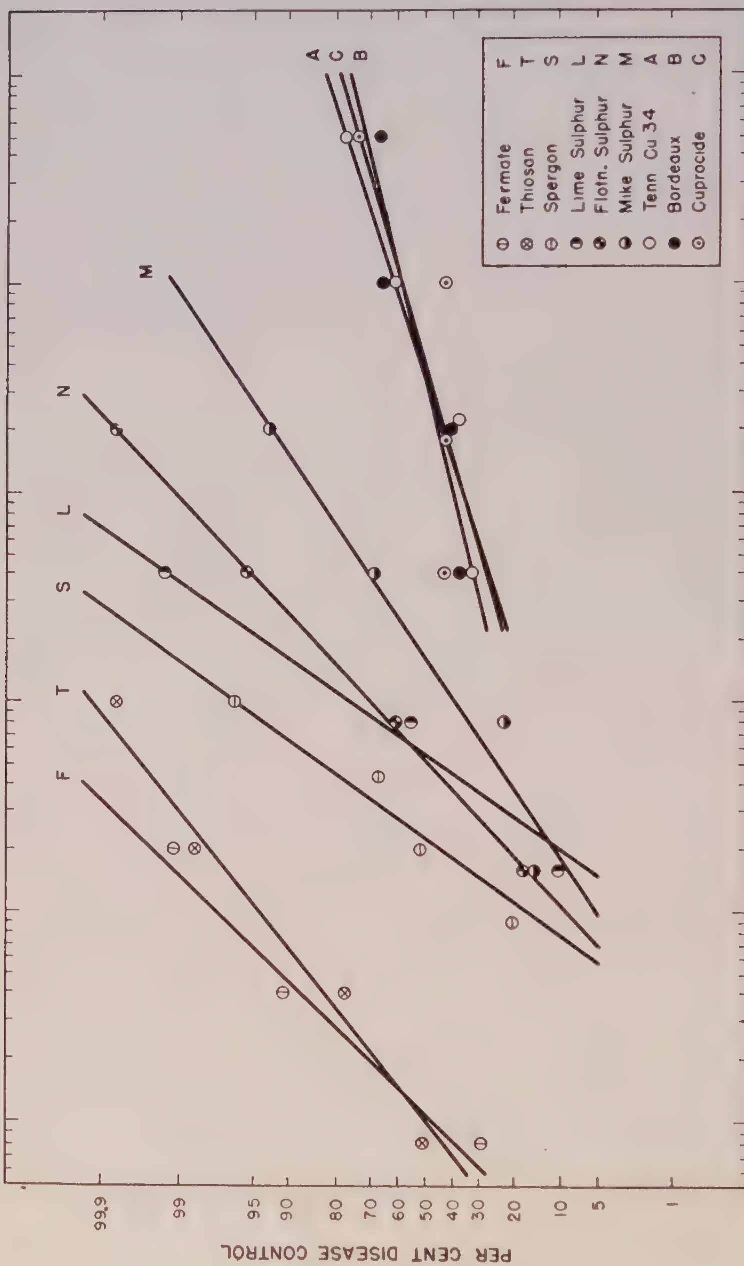

FIGURE 4. Dosage-response curves for organic, sulphur, and copper fungicides against Snapdragon Rust. Note slopes and relative control. Each point mean of two tests with two plants each.

diseases and that copper fungicides can not. The results with the tomato diseases are in marked contrast, the copper fungicides giving good control and the sulphur fungicides being ineffective, which again is in agreement with field results.

The LD95 values in per cent spray dosage, as determined from Figure 4, are recorded in Table VI as well as the per cent disease following a dosage of 0.2 per cent spray. The system of classifying effectiveness of fungicides in exploratory tests previously presented (11) may be extended to include Snapdragon Rust. The organic compounds would receive an AA rating, the sulphur fungicides from AA to B, and the copper fungicides C or D.

COMPARISON WITH SLIDE-GERMINATION TESTS

It was considered of interest to compare the greenhouse disease control response with that of laboratory slide-germination tests with *Puccinia antirrhini*. Spores of *P. antirrhini* were obtained from plants inoculated approximately 20 days earlier, that is, the spores were not more than ten days old. The spores were removed with a camel's hair brush, suspended in distilled water, filtered once through cheesecloth as was customary for the infection tests, and a spore concentration of 50,000 per cc. prepared. Orange juice was not employed as a spore germination stimulant since it was found to be without effect. The nine fungicides were applied to a series of slides by means of the settling tower technique (10) using a dose ratio of 2, and germination tests at 20° C. were performed in the standard manner (1). In all, three separate tests were made and the control percentage germination averaged from about 75 to 80 per cent, for which an appropriate correction was made for potentially viable spores (1, p. 630).

The results of a typical test in which replicate counts were made on a total of 200 spores is shown in Figure 5 which has been drawn to the same relative scale as Figure 4. Due to the overlapping by the copper curves, these were plotted separately in order to give a clearer picture. In comparison with the greenhouse dosage-response curves of Figure 4, it will be seen that the agreement is close in the case of the sulphur fungicides both as regards slope and relative LD value. The organic fungicide curves agree well in relative LD values but are significantly steeper in the laboratory. However, in the case of the copper sprays there is a marked difference; in the laboratory tests they gave a much more effective response and steeper curves. It has been found previously with Early Blight of tomato, caused by *Alternaria solani*, that the laboratory curves are steeper than the greenhouse ones (6).

The comparison of dosages may be put on an absolute basis as has been done in the case of Early Blight. The basic relations found in this earlier case (6, p. 127) can be assumed to hold more or less here, and in any event

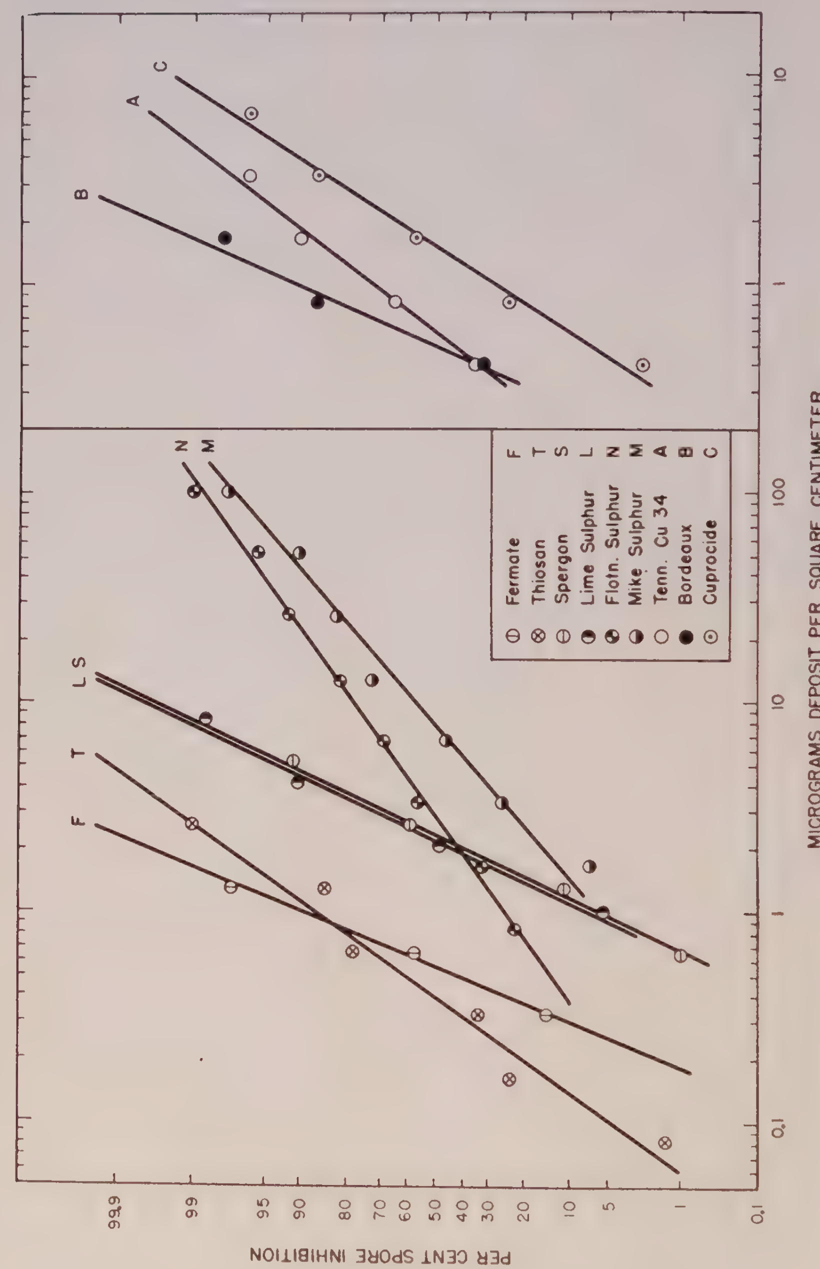

FIGURE 5. Dosage-response curves for organic, sulphur, and copper fungicides against *Puccinia antirrhini* in laboratory slide-germination tests. Compare with greenhouse test in Figure 4.

are likely to be within the error of day-to-day tests. A spore concentration of 100,000 per cc. would be expected to deposit on the leaf at a rate of 170 per sq. cm. In order to obtain the same deposit of spores on glass slides, a concentration of 3,400 per cc. would be required. Assuming the linear function between log. LD and logarithm of spore concentration shown before, the relation between a given LD value for 50,000 and 3,400 spores per cc. is as 1 to 0.45. The absolute dosages thus obtained by these calculations, from the data of Table VI and Figure V, have been summarized in Table VII for the LD95 level. It will be noted that in the case of the sulphur fungicides the LD95 does not differ significantly, with the exception of Flota-

TABLE VII

Comparison of Control in Greenhouse and Slide-Germination Tests with Puccinia antirrhini on Equivalent Basis. LD95 Values in Micrograms Solids Deposited per Sq. Cm. Spore Concentration, 170 per Sq. Cm.

Fungicide	Greenhouse	Slide-germination
Fermate	0.05	0.5
Thiosan	0.08	0.7
Spergon	0.65	2.5
Lime Sulphur*	1.65	2.4
Flotation Sulphur	2.85	18.0
Mike Sulphur	17.25	29.0
Bordeaux Mixture**	>375	0.5
Tennessee Cu "34"	>375	1.1
Cuprocide	>375	2.3

* Micrograms of 32° Baumé stock.
** Micrograms of copper sulphate.

tion Sulphur. That is, the same amount of fungicide is required to prevent the infection of 95 per cent of the spores on leaves as is required to inhibit 95 per cent of the spores from germinating on glass slides. The organic compounds are somewhat more effective on leaves, less being required for the same degree of control, while the copper fungicides shown to be ineffective in greenhouse and field tests are rated very effective in the laboratory slide-germination tests. It is apparent that *Puccinia antirrhini* would markedly over-rate copper fungicides in slide-termination tests. In the case of the comparisons with *Alternaria solani* (6) at the LD95 level, both Thiosan and Tennessee Copper "34," and especially the former, were more effective in the laboratory. In view of all these results it is not possible to generalize as regards efficiency of control or susceptibility of spores on leaves as compared to glass. However, the copper and organic fungicides investigated give steeper curves in the laboratory than in the greenhouse while sulphur gives the same. This perhaps may indicate that the mode of action for copper and organic fungicides differs in the laboratory and greenhouse while for sulphur it is the same.

SUMMARY

1. A greenhouse method has been developed for evaluating foliage fungicides against a representative rust disease, namely Snapdragon Rust.

2. The plants are sprayed and inoculated under controlled conditions with the apparatus previously developed for tomato diseases.

3. The variety Cheviot Maid Supreme was selected as most suitable from among 20 studied, on the basis of susceptibility, growth habit, and availability. The potted plants are trained to twin stems and are at the best stage for testing when 12 inches tall.

4. The total number of pustules per 20 leaves are counted. These leaves are composed of the five adjacent paired leaves on each stem showing the greatest number of pustules. The optimum time for counting is ten days after inoculation.

5. Infection takes place over a fairly wide range of temperature, there being little difference within the range 5° to 20° C., and no significant difference within 10° to 15° C. Satisfactory tests may be made within the former range.

6. Spores are obtained from vigorous unsprayed previously infected plants. A spore concentration of 100,000 per cc. may be expected to produce about 300 pustules per 20 leaves under standard conditions.

7. The number of pustules per paired stem vary less than the number on whole replicate control plants, hence little is gained by counting individual stems. The variation in replicate plants was not reduced by counting the number of pustules per unit area instead of by the simpler standard method of per 20 leaves.

8. In fungicide tests with representative compounds, namely Bordeaux Mixture, Cuprocide, Tennessee Copper "34," Lime Sulphur, Flotation Sulphur, Mike Sulphur, Fermate, Thiosan, and Spergon, the dosage-response curves plotted as straight lines on logarithmic probability paper. Empirical probit weights were obtained which did not differ significantly from those previously calculated for greenhouse tomato diseases. This indicates greatest precision in the vicinity of the LD95 level of control.

9. An analysis of variance on logarithmic LD95 values showed a marked test variation. The replicate plant variation was significantly less and about equal to that of the basic error term, i.e., fungicide × test interaction. One plant per dose ordinarily should suffice, and the tests should be repeated at different times. The method appears equal in precision to that of tomato Early Blight.

10. The organic fungicides gave the steepest dosage-response curves and the most efficient control, the sulphur compounds were intermediate, while the copper fungicide had flat slopes and were ineffective. The results may be expressed preferably as per cent dosage to give 95 per cent control, i.e., the LD95 or as per cent disease at a spray dosage of 0.2 per cent. In

exploratory tests the system of classifying developed for the tomato diseases may be used.

11. Absolute comparisons with laboratory slide-germination tests showed that the sulphur fungicides were rated in the same order and with approximately similar LD95 values and slopes of dosage-response curves. The organic compounds were also rated in the same order but were somewhat more effective in the greenhouse and had steeper curves in the laboratory. The copper fungicides likewise had steeper curves, but were rated very effective in the laboratory.

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QUANTITATIVE STUDIES ON THE PRECIPITIN REACTION OF THE TOBACCO-MOSAIC VIRUS-ANTISERUM SYSTEM[1]

HELEN PURDY BEALE[2] AND MARY E. LOJKIN[3]

INTRODUCTION

Almost half a century ago, Gruber and Durham (9) established the fact that the agglutination of bacteria by homologous antiserum is a constant and specific phenomenon of widespread occurrence. As a logical sequence to this discovery, Kraus (26) observed one year later that a similar reaction, a precipitation, takes place when, instead of the intact typhoid bacillus, cell-free filtrates of broth cultures of this organism are mixed in suitable concentration with the specific antiserum. It was soon universally recognized that practically all proteins assume the rôle of antigens when introduced parenterally into animal tissue, i.e., either intracutaneously, intravenously, intraperitoneally or by some route other than the intestinal tract where their antigenic property is normally lost so that they can no longer stimulate the production of antibody, demonstrable in the blood serum and body fluids of an immunized animal. A further requirement for a true antigen is that it be capable of reacting in some observable way with specific antibody when the two are mixed. Some non-proteinaceous substances such as the polysaccharides of pneumococcus may also function as antigens.

For more than thirty years after the serum-precipitin reaction was first demonstrated, it was of practical value principally as a qualitative test. Within the past fifteen years the quantitative aspects of the reaction, heretofore relatively unreliable, have been extensively investigated (10). To outline the newer method briefly the two reactants, antigen and antibody, are mixed in varying proportions. Usually, the concentration of antibody is kept constant while that of the antigen is increased progressively so that at one end of the reaction range, excess antibody is found in the supernatant after the precipitate has been centrifuged down, whereas at the opposite end of the reaction range antigen is present in excess of that capable of combining with the available antibody. As antigen is successively increased, an inhibition zone is eventually encountered where the precipi-

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tation of an antigen-antibody complex is partially or wholly prevented due to its solubility in excess antigen. A similar inhibition occurs at the opposite end of the reaction range, where excess antiserum is present, but only in horse serums of the antitoxic type (19). Analyses of the specific precipitates have shown that they consist of a mixture of varying proportions of antigen and antibody dependent upon the relative amounts in which they were originally mixed. Since antibody is now generally considered modified globulin and the majority of antigens are also proteins, in determining the composition of the precipitate it is necessary in most cases to distinguish between two proteins, antigen and antibody. In that part of the range of reaction where all the antigen is apparently precipitated, since the amounts of antigen added are known, the amount of antibody present in the precipitate is usually calculated by subtracting the antigen nitrogen from the total nitrogen found in the precipitate. When only part of the antigen is precipitated, the amount of antigen present in the supernatant must also be taken into account before the ratio between antigen and antibody in the precipitate can be determined.

Wu and his collaborators (41) devised methods suitable for determining the composition of the specific precipitate. The first antigen these investigators employed was hemoglobin because a method (40) was available for determining minute quantities of this substance. The total nitrogen in the precipitate which had been washed to remove all non-specific protein was determined by a modified micro-Kjeldahl method. After subtracting the nitrogen attributable to hemoglobin, the remainder was considered antibody nitrogen. Since hemoglobin is a weak antigen, Wu and his co-workers (42) prepared iodoalbumin for the production of antiserum of higher titer. Determinations of both nitrogen and iodine were made on aliquot portions of the washed specific precipitates and the amount of nitrogen contributed by the antigen was calculated from the iodine present while the remaining nitrogen was attributed to antibody.

Heidelberger and Kendall have used various antigen-antiserum systems in detailed studies of the optimal conditions for maximum precipitation and of the composition of the specific precipitates over the entire range of the reaction. As a result of their findings these authors have developed a theory leading to a fuller comprehension of the mechanism of the quantitative precipitin reaction. In one article (12), they described the quantitative precipitin reaction between Type III pneumococcus polysaccharide and purified homologous antibody. The fact that the polysaccharide contained no nitrogen greatly simplified the problem of distinguishing between the two reactants, polysaccharide and antibody. The method subsequently developed and preferred was analysis of nitrogen in the washed precipitate (13). Another article (17) dealt with a proteinaceous antigen to which an azo dye was coupled to enable these investigators to dis-

tinguish between antigen and antibody nitrogen. The amount of dark red azoprotein present in the precipitate was estimated colorimetrically, and subtracted from the total protein in the precipitate to determine the amount of antibody. In a third paper (16), a colorless antigen, crystalline egg albumin, and the homologous antibody were employed. In this instance both antigen and antibody were proteins with no characteristics such as those possessed by hemoglobin, azoprotein, and iodoalbumin, whereby the protein content attributable to antigen could be distinguished from that of antibody. A method described by them in a previous article (13) for determining small amounts of a specific polysaccharide was applied to egg albumin to overcome this difficulty in the zone of excess antigen. An antiserum was first standardized by determining the amount of nitrogen precipitated by known quantities of homologous antigen. The results were incorporated in a curve where the total nitrogen precipitated was plotted against the amount of antigen used. Then a portion of a supernatant containing excess antigen was added to the same amount of antiserum employed in the standardization procedure. Provided the antiserum added was in excess of the amount required to precipitate all of the antigen, unknown quantities of antigen could be determined from the total nitrogen precipitated by reading this value from the curve. In that part of the inhibition zone where the precipitate was partially dissolved in an excess of antigen both reactants, antigen and antibody, were present in the supernatants. The composition of the precipitates in this zone of reaction was also determined by the precipitin reaction using a method of calculation (16, p. 703–704) based upon the assumption that all of the dissolved antibody in the supernatant was precipitated along with that of the added antiserum. While the precipitin method for determining the composition of the precipitates in the complicated region of the inhibition zone was applied with greater facility to the azoprotein-antiserum system, by virtue of the readily distinguishable characteristic of the antigen, than was possible in the case of the colorless egg albumin-antiserum system, the same method of investigation, nevertheless, was found applicable to both.

Culbertson and Seegal (8), in a preliminary report, and Culbertson (7) in a detailed account of their investigations with the crystalline egg albumin-antiserum system concluded that the antigen-dilution method, heretofore largely employed for the titration of precipitable antibody in antiserum, was inaccurate by comparison with their quantitative "neutralization" method and also the microanalytical technic, involving the determination of the composition of the precipitate, as developed chiefly by Wu, and by Heidelberger and Kendall and their respective collaborators. The "neutralization" point of Culbertson and Seegal would be included in the "equivalence" zone of Heidelberger and Kendall. The former authors found the antiserum-dilution method fairly reliable for

comparing the precipitin titers of two or more antiserums but, as they have stated, it does not permit the determination of the total precipitin content of a given volume of antiserum.

No attempt has been made to review all of the literature on the subject of the quantitative serum-precipitin reaction nor to present the various theoretical considerations relative to the mechanism of the reaction since a number of reviews cover this field (4, 5, 10, 11, 24, 27, 31, 32, 35). The purpose of this introduction has been rather to outline the course of development of this more recent and accurate method of studying the reaction between antigens and their precipitins. In the experiments previously referred to, the antigens were all of low molecular weight but the method has also been applied to proteins of high molecular weight such as thyroglobulin (39), various hemocyanins (30), and several plant viruses (25, 37). The investigators of the first two proteins had the advantage of being able to distinguish between antigen and antibody by determining the iodine and copper present respectively in the antigenic fraction of the supernatants and precipitates. In the case of plant viruses the property of inducing disease can be used to detect excess virus in the supernatants.

The present article is the first of a series concerned with the quantitative precipitin reaction of various strains and derivatives of tobacco-mosaic virus and their antibodies. The applicability of the method was first tested on the type strain of tobacco-mosaic virus and its homologous rabbit antibody. That portion of the reaction range from the region of antibody excess through the equivalence zone is covered in this paper. It is hoped that this precise, quantitative precipitin method will prove to be a useful technic for acquiring more information relative to the nature of both antibody and antigen in general and especially the viruses.

EXPERIMENTAL PROCEDURE

MATERIALS AND METHODS

Preparation of antigen. Purified tobacco-mosaic-virus nucleoprotein (*Marmor tabaci* H. var. *vulgare* H.[4]) was kindly supplied by Dr. W. M. Stanley of the Rockefeller Institute for Medical Research in the form of pellets obtained by the ultracentrifugation of a solution of a stock supply. These pellets were dissolved in sterile physiological (0.85 per cent) saline, a drop or two of toluol was added, and the solution was stored in the refrigerator in air-tight stoppered bottles. The final concentration of the virus was approximately 4 mg. per cc. and the pH was 6.00. Micro-Kjeldahl determinations on this preparation performed at intervals over a period of 18 months showed that, under these conditions of storage, the concentration of nitrogen did not become altered. The virus preparation

[4] According to the system of Latin binomials for the designation of viruses as proposed by Holmes (20).

was capable of inducing disease in susceptible plants. Typical symptoms in a tobacco leaf, *Nicotiana tabacum* L., are illustrated in Figure 1 A for comparison with a healthy leaf, B.

Preparation of antiserum. Four rabbits weighing from 3000 to 3750 g. were first given six intravenous injections of antigen followed by five intraperitoneal injections within a period of three weeks. Rabbits Nos. 96, 95, 94, and 93 received a total of 36, 48, 24, and 12 mg. respectively. One rabbit (No. 28) was given a single intravenous injection of 24 mg. From 9 to 10 days after the last injection, the animals were anesthetized and bled to death aseptically from the carotid artery. The serum was separated from the clot and 1 per cent merthiolate was added as a preservative to give a final concentration of 1:10,000. The tubes were plugged with sterile, air-tight stoppers and stored in a refrigerator.

Testing of antigen and antiserum for possible evidence of contamination of the virus preparation with normal-tobacco-plant protein. The virus preparation was tested with antiserum to normal-tobacco-plant protein to detect the possible presence of contaminating normal-plant protein. The antiserums to the virus nucleoprotein were then tested with normal-tobacco-plant protein for the possible presence of precipitin to the plant protein which might have been present had the preparation of purified virus used for the immunization of the rabbits contained any appreciable amount of contaminating plant protein. These tests were extended to include nine antiserums to as many different preparations of various tobacco-mosaic-virus strains, purified by ultracentrifugation in Dr. Stanley's laboratory. The preparation of normal-tobacco-plant protein used for testing the antiserums reacted in an amount as small as 0.02 mg. with a 1:4 dilution of homologous antiserum when the two reactants were mixed, but when the antigen was layered on a 1:1 dilution of the antiserum, a positive ring test was obtained with one-fourth this quantity or 0.005 mg. An evaluation of the results contained in Table I indicates that an amount of normal-plant protein not in excess of 1.5 per cent is present in the virus preparation but no precipitins to this contaminant were demonstrable in the antiserums to the virus.

Quantitative precipitin reaction. The procedure adopted was based largely upon the methods outlined in Heidelberger and Kendall's article. In the majority of precipitin tests a constant amount of antiserum previously diluted was mixed in Wassermann tubes with increasing concentrations of tobacco-mosaic-virus nucleoprotein, the volume being made up in each case to 5 cc. with physiological (0.85 per cent) saline. Volumetric pipettes of the Folin-Ostwald type were used to measure all reagents. The final dilutions of both virus and antiserum were centrifuged prior to performing precipitin tests and duplicate samples of each reactant were transferred to digestion flasks for the determination of their nitrogen con-

TABLE I

TESTS ON TOBACCO-MOSAIC-VIRUS NUCLEOPROTEIN AND THE HOMOLOGOUS ANTISERUM FOR EVIDENCE OF POSSIBLE CONTAMINATION OF THE VIRUS WITH NORMAL-PLANT PROTEIN

Virus nucleoprotein (V) tested with normal serum (NS) and antiserum to normal-plant protein (ANS); antiserum to virus nucleoprotein (AVS) tested with normal-plant protein (N)				
Mg. protein	Cc. undiluted serum	Total vol. cc.	Precipitate*	Ring*
0.384 V	0.125 ANS	2.0	0	
0.000	0.125 ANS	2.0	0	
0.384 V	0.125 NS	2.0	0	
0.000	0.125 NS	2.0	0	
0.818 V	0.100 ANS	0.4	0	+++
0.409 V	0.100 ANS	0.4	0	++
0.205 V	0.100 ANS	0.4	0	0
0.102 V	0.100 ANS	0.4	0	0
0.051 V	0.100 ANS	0.4	0	0
0.025 V	0.100 ANS	0.4	0	0
0.000 V	0.100 ANS	0.4	0	0
0.409 V	0.000	0.4	0	0
0.818 V	0.100 NS	0.4	0	0
0.409 V	0.100 NS	0.4	0	0
0.000 V	0.100 NS	0.4	0	0
0.035 N	0.10 AVS	0.4	0	
0.018 N	0.10 AVS	0.4	0	
0.009 N	0.10 AVS	0.4	0	
0.000	0.10 AVS	0.4	0	
0.070 N	0.00	0.4	0	
0.035 N	0.00	0.4	0	
0.018 N	0.00	0.4	0	
0.009 N	0.00	0.4	0	

Normal serum (NS) and antiserum to normal-plant protein (ANS) tested with normal-plant protein (N)				
Mg. normal protein	Cc. undiluted serum	Total vol. cc.	Precipitate*	Ring*
0.16	0.30 ANS	0.6	++++	
0.16	0.15 ANS	0.6	+++	
0.16	0.08 ANS	0.6	0	
0.08	0.06 ANS	0.6	+++	
0.04	0.06 ANS	0.6	++	
0.02	0.06 ANS	0.6	+	
0.01	0.06 ANS	0.6	0	
0.00	0.06 ANS	0.6	0	
0.08	0.00	0.6	0	
0.070	0.10 ANS	0.4	++++	
0.035	0.10 ANS	0.4	+++	
0.018	0.10 ANS	0.4	+	
0.009	0.10 ANS	0.4	±	
0.070	0.10 NS	0.4	0	
0.035	0.10 NS	0.4	0	
0.037	0.05 ANS	0.4	++	++
0.019	0.05 ANS	0.4	+	+
0.009	0.05 ANS	0.4	±	+
0.005	0.05 ANS	0.4	0	+
0.002	0.05 ANS	0.4	0	±
0.037	0.07 NS	0.4	0	0
0.074	0.00	0.4	0	0
0.037	0.10 ANS	0.4	++	+++
0.019	0.10 ANS	0.4	+	++
0.009	0.10 ANS	0.4	0	+
0.005	0.10 ANS	0.4	0	±
0.000	0.10 ANS	0.4	0	0
0.037	0.00	0.4	0	0

* 0 = no precipitate; ± = very slight precipitate; + = slight precipitate; ++ = moderate precipitate; +++ = heavy precipitate; ++++ = very heavy precipitate. The same symbols are used for the "ring" test with reference to the amount of precipitate formed at the antigen-antiserum interface when antigen is layered upon antiserum.

tent by the micro-Kjeldahl method. Preliminary experiments in which the tubes were held in the refrigerator without incubation in a water-bath, or tests in which the tubes were first incubated at 37° C. for one to two hours followed by refrigeration proved that these conditions were not so favorable for the formation of the maximum precipitate as the procedure

adopted eventually of incubation in a water-bath at 45° to 47° C., followed by refrigeration for 48 hr., and finally freezing the contents of the tubes for 20 hr. or longer. For incubation the water level in the bath was regulated to insure the immediate setting up of convection currents which insured a thorough mixing of the contents and consequently facilitated the formation of the maximum precipitate. Freezing appeared to cause the characteristically bulky, flocculent precipitates to pack more firmly so that there was less possibility of losing floccules when decanting the supernatant fluids during the washing of the precipitates. After incubation, refrigeration, and freezing, the contents of the tubes were thawed and the precipitates were centrifuged down at high speed for 30 min. The supernatants were carefully decanted into other tubes and stored in the refrigerator for future testing, while the precipitates were washed to free them of extraneous proteinaceous material. Throughout the entire procedure, the contents of the tubes containing precipitates were kept at 0° to 5° C. by packing the centrifuge carriers containing the tubes in cracked ice Since no brine-cooled centrifuge was available, a portable centrifuge was transferred to a cold room at a temperature below freezing. The carriers were kept in an insulated metal box filled with chipped ice and fitted with a cover containing openings just large enough to allow immersion of the carriers in the ice. The box was mounted on a mobile stand so that it could be wheeled back and forth from the cold room to the laboratory for alternate centrifuging and washing of the precipitates. The precipitates were washed by adding 2 cc. of chilled physiological saline to each tube and gently agitating the contents to loosen and homogenize the precipitate packed in the bottom. These suspensions were allowed to stand chilled for 30 min., then they were centrifuged for 20 min. After decanting the saline washes, the tubes were inverted to allow drainage of the remaining liquid. The mouths of the tubes were wiped with strips of filter paper before adding fresh saline. Inversion of the tubes was not practical until after the first washing due to the likelihood of losing precipitate. The entire procedure of washing the precipitates was performed three times. Included in this operation were control tubes which had contained only the virus or antiserum. The washes from all tubes were set aside for future testing. After the final washing was completed, N/10 NaOH was added to each tube and the contents were transferred to digestion flasks. Micro-Kjeldahl determinations of the nitrogen present were made following Pregl's method (36) with modifications, using the distillation apparatus of Parnas and Wagner (33) and titrating with N/70 hydrochloric acid. The amount of N found was translated into terms of protein by multiplication with 6.25.

Testing of supernatants for excess reactant. Separate portions of the supernatants obtained from the first decantation were mixed with anti-

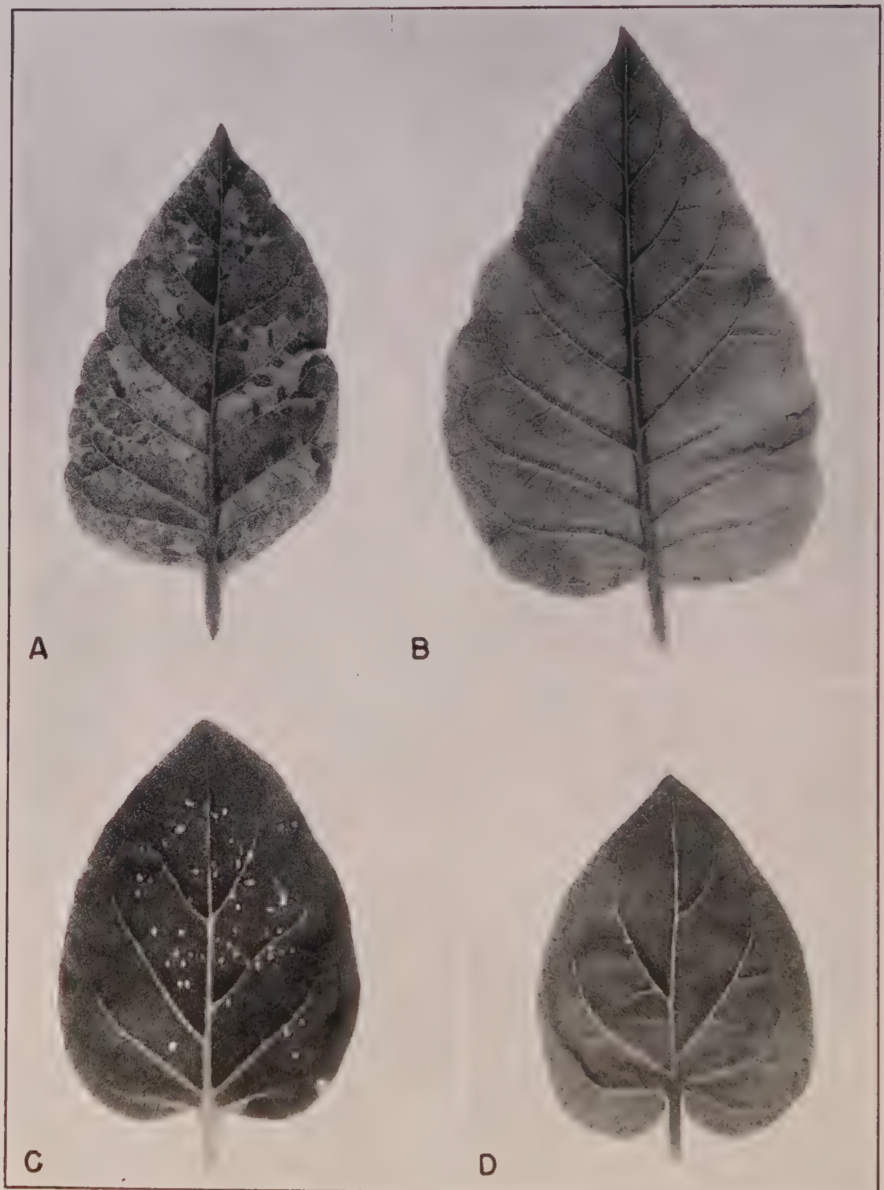

FIGURE 1. Leaves from healthy and virus-infected plants. A. Tobacco, *Nicotiana tabacum* L., showing mottling typical of a systemic infection with tobacco-mosaic virus. B. Healthy tobacco. C. *Nicotiana glutinosa* L. showing local necrotic lesions, developed after rubbing the surface of the leaf with a cheesecloth pad moistened with tobacco-mosaic virus. D. Healthy *N. glutinosa*.

serum or virus in order to determine which, if either, component was present in excess. Other portions of these supernatants were inoculated both undiluted and in various dilutions on leaves of a susceptible plant, *Nicotiana glutinosa* L., so that an excess of active virus present could be detected through the development of local necrotic lesions apparent within three to five days (Fig. 1 C). An uninoculated leaf (Fig. 1 D) is shown for comparison. In the majority of cases, the saline solutions decanted after the first, second, and third washings of the precipitates were inoculated on susceptible leaves in an attempt to detect possible dissociation

TABLE II

EFFECT OF NORMAL SERUM ON NUMBERS OF LOCAL LESIONS INDUCED IN NICOTIANA GLUTINOSA L. BY INOCULATION WITH SERUM-VIRUS MIXTURES

Cc. of serum in cc. of virus concn. of 0.10 mg./cc.	Total No. lesions	Diff. between No. lesions induced by virus-saline and virus-serum mixtures	No. lesions calculated from regression equation† $Y=964.894-329.0\,X$
0.001	999	7	965
0.002	850	156	866
0.005	699	307**	735
0.010	655	350**	636
0.013	571	435**	598
0.040	463	543**	438
0.000	1006	000	—

† Regression of number of lesions on logarithm of concentration of serum.
** Significant at odds of 99 :1, requiring a difference of 295 lesions (38).

or solution of the precipitate during the washing operations. The washes from the control tubes which contained virus and no antiserum were also inoculated on susceptible leaves.

Determining concentration of serum which will not exert an inhibitory effect on virus infectivity. A number of investigators have shown that when rabbit serum is added to tobacco-mosaic virus in sufficient concentration, the infectivity of the virus is diminished, as evidenced by a reduction in the number of local necrotic lesions developing subsequent to inoculation of the mixture on *N. glutinosa* (Fig. 1 C). Consequently, in experiments where whole antiserum is used instead of a purified antibody solution, it is necessary to dilute the supernatants prior to plant inoculation in order to overcome this inhibitory effect. Therefore, an experiment was planned to determine the concentration of serum which will not affect virus infectivity appreciably. A single series of tests was made in which increasing concentrations of normal-rabbit serum were mixed with a constant concentration of virus, and these mixtures were held at room temperature for four hours, placed in the refrigerator overnight, and then inoculated separately on leaves of *N. glutinosa*. For the purpose of comparison, a sample of virus containing physiological saline in place of serum was also

included in the tests. The virus concentration selected for this experiment was such that it exhibited an infectivity which various investigators (3, 28) have shown previously to be within the most sensitive range for ascertaining differences in amounts of active virus. The inoculations were made according to the Latin-square arrangement which has been applied successfully in this field (45).

DISCUSSION AND RESULTS

The inhibitory effect of normal serum on virus infectivity is shown in Figure 2, where the regression of the number of lesions on the logarithm

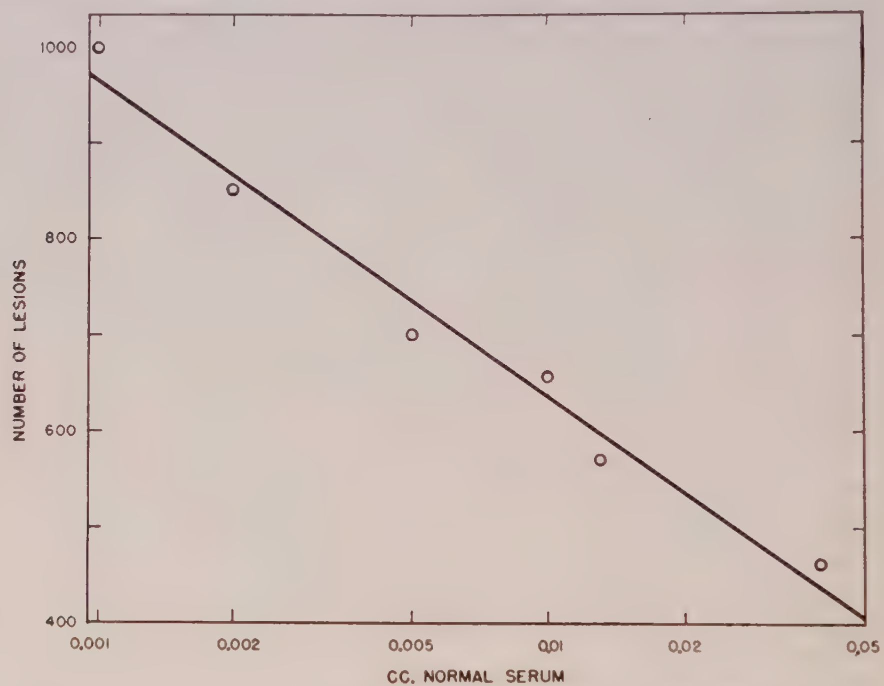

FIGURE 2. Inhibitory effect of increasing concentrations of normal-rabbit serum on a constant concentration of virus, as shown by the number of local lesions developed after inoculation of the virus-serum mixtures on *Nicotiana glutinosa* L. (Fig. 1 C). Regression of number of lesions on logarithm of concentration of normal serum. $Y = 964.894 - 329.0\ X$.

of the concentration of normal serum shows close correlation between the two variables. The experimental values show close agreement with those calculated from the regression equation (Table II). The total number of lesions resulting from the inoculation of seven leaves each with the various virus-serum mixtures is compared with the corresponding number induced by the control virus-saline mixture. By analysis of variance, the numbers of lesions required for a difference to be significant were cal-

culated to be 295 at odds of 99:1, and 219 at odds of 19:1 (38). From the table, it will be noted that for dilutions ranging from 0.005 to 0.040 cc. of serum per cc. of virus-saline solution, the odds are 99:1 that the inhibitory effect indicated is significant, while the presence of 0.002 cc. of serum per cc. in the virus mixture exerted little inhibitory effect and a concentration of 0.001 cc. of serum per cc. produces no demonstrable effect and is indistinguishable from the virus-saline control containing no serum. These results agree well with those of Chester (6, p. 1184), who after a detailed study of the inhibitory effect of serum on tobacco-mosaic virus, recommends diluting the serum to at least 0.001 per cc. to minimize this effect rather than using the higher concentration of 0.01 per cc. generally employed by him for virus preparations of low potency. Since the majority of supernatants used for plant inoculation in the experiments reported in Table III contained either no virus or only traces, they were usually tested at serum concentrations of 0.001 per cc. and higher. This inhibitory effect of serum on virus infectivity detracts from the value of the plant method as a means of detecting virus in supernatants containing whole serum. On the other hand, the serum-precipitin method is very delicate since during the course of these investigations, when known quantities of virus were added to the homologous antiserum, a small amount of precipitate was obtainable with an amount of virus equal to 0.0003 mg. Also the virus may lose its infectivity and still form a precipitate with antibody (2, p. 118).

The results of the plant inoculations with the washes from the precipitates (Table III) indicate that the latter are practically insoluble in chilled saline and apparently do not dissociate to any extent. The results of tests on the washes from the virus-saline controls, not included in the tables, show that the largest number of lesions was induced with the first wash, a smaller number with the second, and generally none with the third wash, which demonstrates the efficacy of the method of washing.

The precipitin experiments with antiserums, Nos. 94 and 96, are reported in this article, since only they are sufficiently extensive to test the applicability of the quantitative serum-precipitin method to the tobacco virus-antiserum system. Many of the determinations included on antiserum No. 94 are preliminary and, therefore, not so consistent or accurate as those made later on antiserum No. 96 when the experimental procedure had been better standardized. A brief report of the results of the tests with antiserum No. 94 is included, however, since this antiserum was tested over a wider range of virus concentrations. Total amounts varying from 0.053 to 2.194 mg. virus were used with this antiserum compared to 0.074 to 0.622 mg. in the case of antiserum No. 96. Although the discussion is confined mainly to the region of antibody excess and a portion of the equivalence zone, the experimental results over the entire reaction range

investigated are included in Table III and Figures 3 and 7. The three zones comprising the entire range of the precipitin reaction, those of antibody excess, equivalence, and antigen excess, are separated from each other by double lines, as they appear in this order in the table. The experimental data are represented graphically in Figures 3, 4, and 5.

The results show, in general, that when increasing amounts of virus are added to a constant amount of antiserum, the total virus-antibody

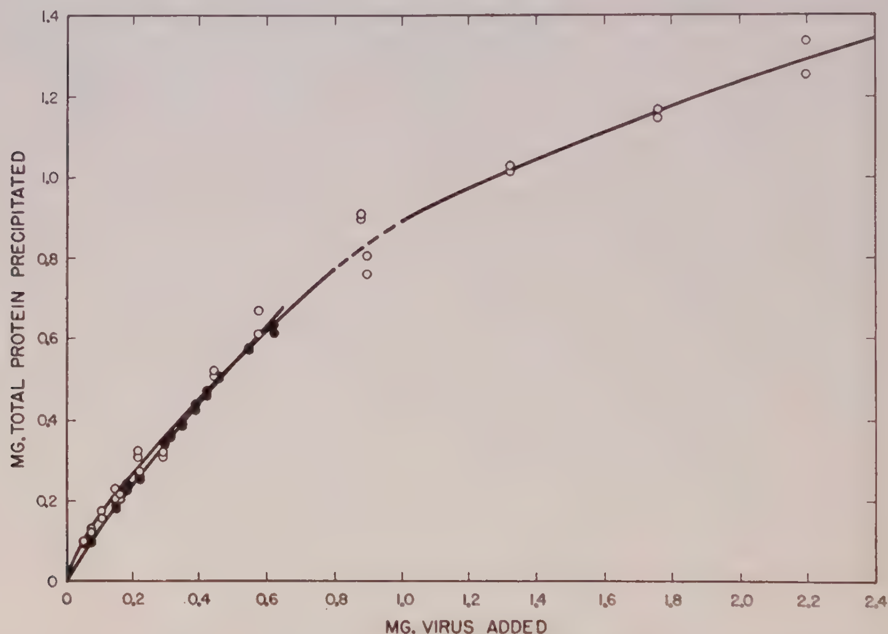

FIGURE 3. Relationship between mg. virus added and amounts of precipitate formed when increasing quantities of virus are added to a constant amount of antiserum. Curves are drawn according to the regression lines, calculated for the logarithms of Y on the logarithms of X. Antiserum No. 94, light circles; antiserum No. 96, dark circles. The dashed line signifies a break in the curve which necessitated the calculation of two separate regression lines, so that for amounts of virus from 0.053 to 0.895 mg., $Y=0.445+0.759\ X$; and, from 0.895 to 2.194 mg., $Y=0.999+0.475\ X$. For antiserum No. 96, $Y=0.264+0.863\ X$.

precipitate formed increases successively throughout the regions of antibody excess and equivalence and through that part of the region of antigen excess investigated which did not include the inhibition zone. The results of the precipitin reactions are presented graphically in Figure 3, where the total protein precipitated is plotted as ordinates against the virus added as abscissae, the light circles indicating the experimental values for antiserum No. 94, and the dark circles those for antiserum No. 96. The curves are drawn according to the values obtained by calculating the regression

of the logarithms of the Y-axis on the logarithms of the X-axis. A break in the curve for antiserum No. 94, which is indicated by the dashed line, necessitated the calculation of two separate regression lines, one for amounts of virus from 0.05 to 0.90 mg. and another for amounts ranging from 0.88 to 2.19 mg. It is of some interest that the break occurred at the end of the equivalence zone and at the beginning of the region of antigen excess, although the significance of the observation is not clear. In the case of both antiserums, the composition of the precipitates varies accord-

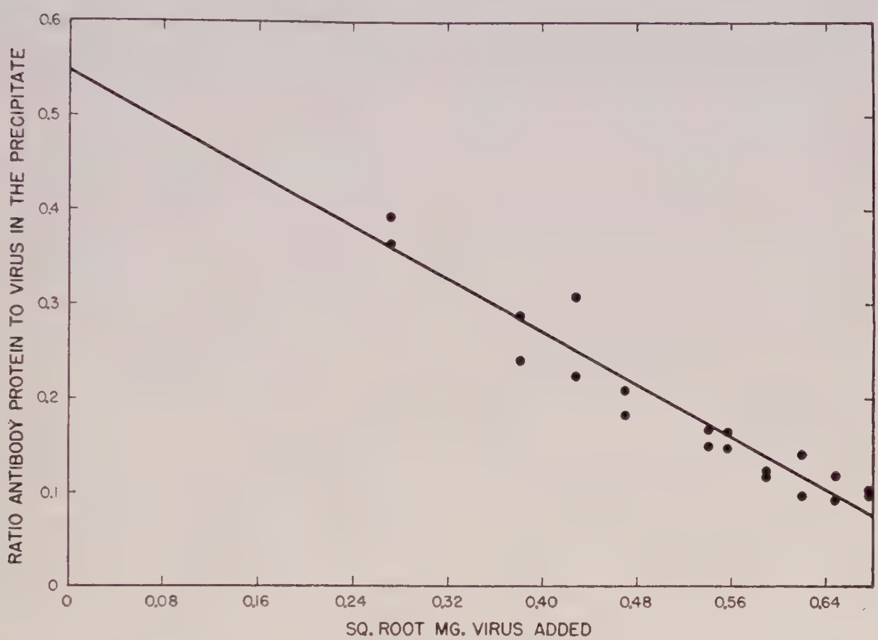

FIGURE 4. Relationship between ratio of antibody protein to virus in the precipitate and the square root of mg. virus added. Antiserum No. 96; $Y = 0.543 - 0.688\ X$.

ing to the proportions in which the virus and the homologous antiserum are mixed. The amount of antibody in the precipitates from antiserum No. 96 (Table III) increases at first in the region of antibody excess and tends to remain at a fairly constant level throughout most of the equivalence zone. It is shown from the data in Table III and from Figure 5 that when the ratios of antibody to virus in the precipitates are plotted as ordinates against the amounts of virus added as abscissae, the ratios in general decrease successively in the region of antibody excess and in the adjacent portion of the equivalence zone.

In accord with other investigators of high-molecular-weight antigens, such as hemocyanin (30) and thyroglobulin (39) for which ratios of anti-

TABLE III

DATA ON THE PRECIPITIN REACTION BETWEEN INCREASING AMOUNTS OF TOBACCO-MOSAIC VIRUS AND 0.33 CC. OF THE HOMOLOGOUS ANTISERUM, NO. 96

Test No.	Total virus added, mg.	Total protein precipitated, mg.	Antibody protein by difference (col. 3–col. 2), mg.	Ratio of antibody protein to virus in precipitate	Excess antibody or virus in supernatant as determined by serum-precipitin reaction	Excess virus in supernatant or washes as determined by plant inoculation: Cc. of serum in cc. of diluted supernatant	Av. No. lesions per leaf: Supernatant	Washes: 1	Washes: 2	Washes: 3
1	0.074	0.101	0.027	0.36	Antibody	0.005	0	0	0	0.5
		0.103	0.029	0.39		0.001	0			
2	0.146*	0.181	0.035	0.24	Antibody	0.005	0	0	0	0
		0.188	0.042	0.29		0.001	0			
3	0.183	0.239	0.056	0.31	Antibody	0.005	0	0	0	0
		0.224	0.041	0.22		0.001	0			
4	0.220*	0.260	0.040	0.18	Antibody	0.005	0	0	0	1.3
		0.266	0.046	0.21		0.001	0			
5	0.293	0.337	0.044	0.15	Antibody	0.005	0	0.5	0.5	0.5
		0.342	0.049	0.17		0.001	0			
6	0.310	0.356	0.046	0.15	None	0.005	0	0	1.5	0
		0.361	0.051	0.17		0.001	0			
7	0.347	0.390	0.043	0.12	None	0.005	0	0	0.5	1.0
		0.388	0.041	0.12		0.001	0			

TABLE III (*Continued*)

Test No.	Total virus added, mg.	Total protein precipitated, mg.	Antibody protein by difference (col. 3–col. 2), mg.	Ratio of antibody protein to virus in precipitate	Excess antibody or virus in supernatant as determined by serum-precipitin reaction	Excess virus in supernatant or washes as determined by plant inoculation				
						Cc. of serum in cc. of diluted supernatant	Av. No. lesions per leaf			
							Supernatant	Washes		
								1	2	3
8	0.384*	0.421	0.037	0.10	None	0.005	0	0.25	1	3.5
		0.438	0.054	0.14		0.001	0			
9	0.420	0.470	0.050	0.12	None	0.005	0	0	1	0.5
		0.459	0.039	0.09		0.001	0			
10	0.457*	0.502	0.045	0.10	None	0.005	0	0	3.25	1.3
		0.504	0.047	0.10		0.001	0			
11	0.548*	0.575	0.027	0.05	None	0.005	0	0	3.8	2.8
		0.572	0.024	0.04		0.001				
12	0.622*	0.633	—	—	Virus	0.005	0	0	1.8	1.8
		0.614*	—	—		0.001	0			

* Average of two determinations.

body to antigen in the precipitates may fall to less than unity, the ratios (Table III) in or near equivalence for the virus-antiserum system are all less than unity and may be as small as the second decimal place. Considering the large molecular weight of the virus, estimated to be about 50,000,000 (23) compared to that determined for rabbit antibody of approximately 160,000 (18, 22), low ratios are to be expected.

It will be noted (Table III, columns 6 and 8) that all of the virus is completely precipitated in the zones of antibody excess and equivalence; therefore the practice adopted of determining the antibody content of the specific precipitates formed in this region of the reaction by subtracting the virus added from the total protein precipitated, as indicated in column 4, is considered suitable. The tests also reveal no appreciable amounts of either reagent, virus or antibody, in the supernatants of the equivalence zones. The fact that both reagents are almost completely precipitated from the supernatants of this region has been regarded by investigators of some other antigen-antiserum systems as evidence that the antigen is reacting as a single component (21, p. 230; 39, p. 258). The fact that the small amount of normal-tobacco-plant protein, known to be present in the virus preparations, apparently does not enter into the reaction would appear to be due to the immunization procedure whereby only small amounts of virus, totaling 24 and 36 mg., were injected within a single period of no more than three weeks' duration.

Because of the convenience of the method, considerable time was expended on comparing various means of representing the data by as strictly a linear relationship as possible. Some equations derived by Heidelberger and Kendall (14, p. 587–588; 15, p. 479), and applicable to the virus-antiserum system, result in a straight line useful for the evaluation of certain constants by means of which it is possible to determine important characteristics of a given antiserum, such as the maximum precipitable antibody and the ratio of antibody to antigen in the precipitate at this maximum. The more accurately this relationship can be expressed, the greater will be the precision in interpreting the results and the sounder the basis for future consideration of the theoretical aspects of the problem.

A comparison is made (Table IV, columns 2 to 5) of the experimental values obtained for the amounts of antibody in the precipitates in the regions of antibody excess and adjoining portions of equivalence with those calculated from the linear regression equations by the method of least squares (34, p. 134), based upon the same experimental points plotted in three different ways, arithmically, semi-logarithmically, and logarithmically (Fig. 5). As indicated by the variances recorded in the table, no closer agreement is obtained than that between the observed values and those calculated from the semi-logarithmic regression line, when the ratios of antibody to virus in the specific precipitates is plotted on the logarithms

TABLE IV

COMPARISON OF THE AMOUNTS OF ANTIBODY PROTEIN FOUND IN THE PRECIPITATES WITH THOSE CALCULATED FROM VARIOUS EQUATIONS. MG. ANTIBODY PROTEIN PRECIPITATED FROM 0.33 CC. ANTISERUM, NO. 96, BY INCREASING AMOUNTS OF TOBACCO-MOSAIC VIRUS

Virus added, mg.	Antibody protein found in precipitate†, mg.	Regression equations††			Equations‡ derived by Heidelberger and Kendall	
		Arithmic $Y=0.383-0.692X$	Semi-logarithmic $Y=0.697-0.365X$	Logarithmic $Y=2.310-0.774X$	Mg. antibody precipitated = $0.313V-0.025V^2$	$0.543V-0.688V^{3/2}$
0.074	0.028	0.025	0.028	0.032	0.020	0.026
0.146‡‡	0.039	0.041	0.040	0.038	0.035	0.041
0.183	0.049	0.047	0.043	0.039	0.040	0.046
0.220‡‡	0.043	0.051	0.046	0.041	0.044	0.049
0.293	0.047	0.053	0.047	0.044	0.048	0.050
0.310	0.049	0.052	0.047	0.044	0.049	0.050
0.347	0.042	0.050	0.047	0.045	0.040	0.048
0.384‡‡	0.046	0.045	0.046	0.046	0.046	0.045
0.420	0.045	0.039	0.044	0.047	0.042	0.041
0.457‡‡	0.046	0.031	0.042	0.048	0.038	0.036
Variance		0.000050	0.000010	0.000019	0.000027	0.000024
Ratio arithmic variance to other variances			5*	2.6	1.9	2.1

† Average of duplicate determinations (see Table III, column 4).

†† Regression of ratio antibody protein to virus in the precipitate (Y-axis) on mg. virus added (X-axis); (semi-logarithmic) regression of Y on logarithm of X.

‡ Equation in column 6 (14, p. 587–589); equation in column 7 (15, p. 479).

‡‡ See Table III, footnote *.

* Significant at odds of 19 :1, requiring a ratio of 4.1 or greater (29).

of the amounts of virus precipitated. Reference to McCallan and Wellman's table (29, p. 138) computed from Bartlett's test for homogeneity of variance (38, p. 206) shows a significant improvement in favor of the semi-logarithmic method over the arithmic. It is evident that the experimental points at extreme antibody excess would fall closer to a curve than to the arithmic regression line. The exact reason for this deviation is not known, but a possible explanation is that the surface area of the virus has been increased either through the dissolution of particle aggregates or by

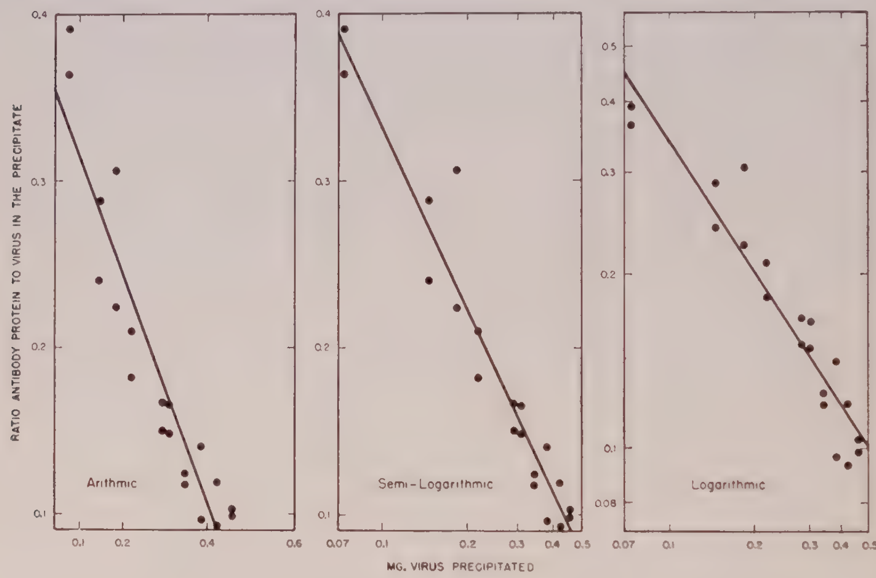

FIGURE 5. Relationship between ratio of antibody protein to virus in the precipitate and mg. virus precipitated. Data for antiserum No. 96 in zones of antibody excess and more than half of equivalence, plotted various ways to obtain the best linear representation. $Y=0.383-0.692\ X$ (arithmic), $Y=0.697-0.365\ X$ (semi-logarithmic), $Y=2.310-0.774\ X$ (logarithmic).

dissociation so that at the lower concentrations a larger reactive surface becomes available for combination with antibody. A similar behavior of the virus was encountered by Bald (1, p. 75) and by Youden (44, p. 51–55) in studying the relation of dilution to numbers of lesions induced subsequent to plant inoculation. Instead of a consistent decrease in the numbers of lesions generally accompanying increasing dilution, when the concentration of virus reached an amount of about 0.1 mg. per cc., there was not only no reduction in the number of lesions but there was considerable evidence that an increase occurred. Also, Wyckoff, Biscoe, and Stanley (43) in an ultracentrifugal analysis of the virus state that solutions of tobacco-mosaic virus diluted to 0.5 mg. per cc. show a molecular disso-

TABLE V

COMPARISON OF THE AMOUNTS OF ANTIBODY NITROGEN FOUND IN THE PRECIPITATES WITH THOSE CALCULATED FROM VARIOUS EQUATIONS. MG. ANTIBODY NITROGEN PRECIPITATED FROM 1.00 CC. ANTISERUM BY INCREASING AMOUNTS OF THYROGLOBULIN

Thyroglobulin added†, mg.	Antibody nitrogen found in precipitate†, mg.	Regression equations††			Equations† derived by Heidelberger and Kendall‡	
		Arithmic $Y=5.788-10.827X$	Semi-logarithmic $Y=11.303-5.982X$	Logarithmic $Y=1.615-0.833X$	Mg. antibody N precipitated = $6.5\ Tg.N-15.0\ (Tg.N)^2$	$8.0\ Tg.N-10.4\ (Tg.N)^{3/2}$
0.144	0.640	0.609	0.630	0.644	0.625	0.582
0.216	0.698	0.745	0.716	0.688	0.704	0.682
0.288	0.730	0.769	0.742	0.724	—	—
0.316	0.727	0.748	0.736	0.734	—	—
0.364	0.749	0.672	0.715	0.752	—	—
Variance		0.002765	0.000451	0.000051	0.000261	0.003620
Ratio arithmic variance to other variances			6.1	54.2*	10.6	0.8
		$Y=4.613-13.969X$	$Y=6.545-3.642X$	$Y=1.008-0.577X$	$4.5\ Tg.N-14\ (Tg.N)^2$	
0.040	0.173	0.162	0.174	0.183	0.158	
0.079	0.263	0.277	0.259	0.244	0.269	
0.158	0.340	0.380	0.344	0.328	0.361	
0.190	0.352	0.372	0.359	0.354	—	
0.237	0.373	0.309	0.364	0.389	—	
Variance		0.001603	0.000041	0.000216	0.000351	
Ratio arithmic variance to other variances			39.1*	7.3	4.6	

† Taken from Stokinger and Heidelberger (39, p. 261–262, Table IV). Human Tg. (first five tests); beef Tg. (second five tests).

†† Regression of ratio antibody N to thyroglobulin N in the precipitate (Y-axis) on mg. thyroglobulin N added (X-axis); (semi-logarithmic) regression of Y on logarithm of X.

‡ See footnote ‡, Table IV.

* Significant at odds of 19 :1, requiring a ratio of 9.7 or greater for 4 D.F. (29).

ciation. Digression from a strictly linear relationship has been reported in the zone of antibody excess by investigators of another high-molecular-weight protein, thyroglobulin. The authors themselves were well aware of the fact that when the results were plotted arithmically, connecting the actual experimental points, in most cases, as stated by them, "led to a curve and not a straight line" (39, p. 260). Here, also, the relationship between antibody and antigen in the precipitate can be better expressed as linear by plotting the data not arithmically, but either semi-logarithmically or logarithmically. Two examples of such data chosen at random are reproduced in Table V. As indicated by the variances, the regression equations giving results in closest agreement with the observed values are the logarithmic for one set of data and the semi-logarithmic for the other shown in Figure 6, where the regression of the ratios of antibody to thyroglobulin in the precipitate was plotted on the logarithms of the amounts of thyroglobulin added. The significance of the variances was calculated by the method used for antiserum No. 96.

A fairly good linear relationship can also be represented graphically, for the virus-antiserum results, as demonstrated by Heidelberger and Kendall for their data (15, p. 479), by plotting the ratios of antibody to virus comprising the precipitate against the square roots of the amounts of virus added, as in Figure 4. Furthermore, these authors derived an equation based upon this treatment of the data whereby they found it possible to perform as few as two or three analyses, draw a line through these points, and then they could ascertain quite accurately the behavior of the antiserum under investigation over a wide range by substituting the values indicated by the intercept on the Y-axis in the equation. According to their equation:

$$\frac{\text{antibody } N}{D} \text{ in the precipitate} = 3R'' - 2\sqrt{\frac{(R'')^3}{A}D} \qquad \text{(Equation 1)}$$

in which $3R''$ = the intercept on the Y-axis, $-2\sqrt{\frac{(R'')^3}{A}D}$ = the slope of the line, D = the amount of antigen precipitated, A = the maximum precipitable antibody nitrogen, R'' = the ratio of A to D at the maximum. When this equation is applied to the experimental data herein presented, the calculated values for the amounts of antibody precipitated are in agreement with the experimental values found (Table IV, column 7). The data in this article are expressed in terms of protein instead of nitrogen as used in the equation. Also, the regression lines are calculated from all of the experimental determinations available (Fig. 4) rather than using only two or three in the region of antibody excess which is recommended

as practical. The maximum precipitable antibody protein as calculated for antiserum No. 94 is 0.072 mg. per 0.33 cc. The maximum precipitable antibody in 0.33 cc. of antiserum No. 96 is estimated from the equation as 0.050 mg. compared to the experimental value of 0.049 mg. (Table IV). The ratio of antibody to virus calculated at the point of maximum precipitable antibody for antiserum No. 96 is 0.18 compared to the observed

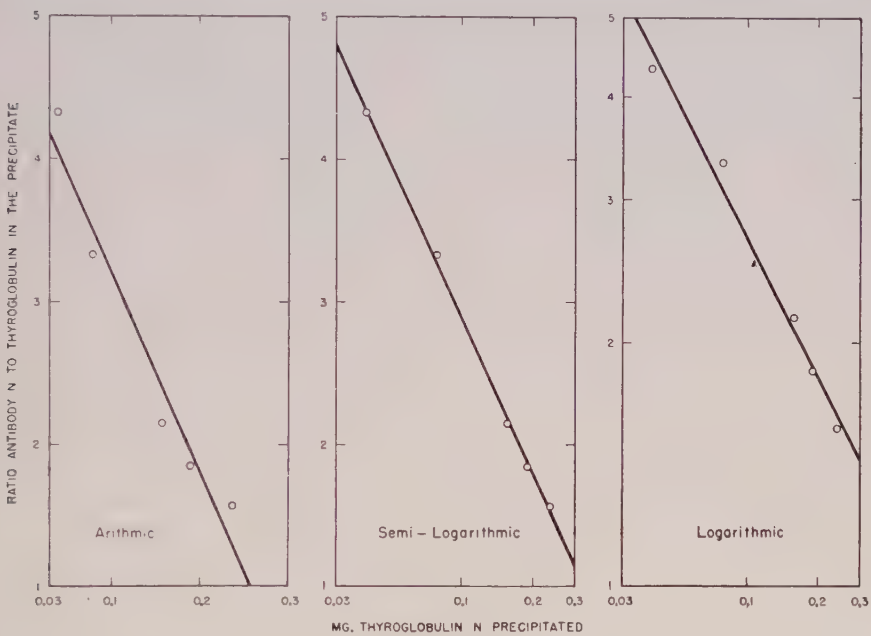

FIGURE 6. Relationship between ratio of antibody N to beef thyroglobulin N in the precipitate and mg. thyroglobulin N added. From Stokinger and Heidelberger (39, p. 262, Table IV). Data plotted in various ways to obtain the best linear representation. $Y=4.613-13.969\ X$ (arithmic), $Y=6.545-3.642\ X$ (semi-logarithmic), $Y=1.008-0.577\ X$ (logarithmic).

value of 0.19, representing the average of four ratios in duplicate included within the zone of maximum precipitation (Table III, column 5, tests 3 to 6).

In the case of antiserum No. 94 the amounts of antibody precipitated in the region of antiserum excess and the beginning of the equivalence zone agree well with values calculated according to another equation derived by Heidelberger and Kendall (14, p. 587–589):

$$\frac{N}{S}=2R-\frac{R^2}{A}S \qquad \text{(Equation 2)}$$

where S corresponds to the virus nucleoprotein (V) added and A is equal to the amount of antibody precipitated, and where values for the ratio of antibody to virus in the precipitate (R) found in the region of excess antibody are plotted as ordinates against the amounts of virus added as abscissae. The intercept on the Y-axis gives the value $2R$, while the slope of the line is $\frac{R^2}{A}$. For the regression line representing these points, the values for A calculated from equation 2 are practically identical to those obtained from the arithmic regression equation. In Table IV, column 6, equation 2 has been applied somewhat differently from the method just described. The average of the duplicate values for R, observed for the first point in the equivalence zone (Table III, column 5, test 6) was substituted for R in the equation, while the average of the duplicate analyses of the amounts of antibody precipitated at this same reference point was taken for A. This application of the equation is also recommended and made use of by Heidelberger and Kendall (14, p. 584–585) in their own work.

COMPARISON OF DATA WITH THAT OF OTHER INVESTIGATORS IN THE FIELD

Kleczkowski (25) produced several rabbit antiserums to tobacco-mosaic virus but the one of highest titer (approximately 0.2 mg. per 0.33 cc.) was obtained from the animal receiving the largest amount of virus and the greatest number of injections over the longest period of time, i.e., 24 mg. of virus administered in six doses within three weeks. Practically the same immunization procedure was used for the production of antiserums, Nos. 94 and 96, as described previously in more detail. Schramm and Friedrich-Freska (37) injected a rabbit with a total of 130 mg. of virus in five portions within seven weeks, then after a rest interval of five months the animal was again injected twice with 30 mg. additional virus. With this prolonged immunization procedure during which such large amounts of virus were injected, qualitative as well as quantitative differences in antibody might be expected. It is of interest to note here that neither Kleczkowski nor Schramm and Friedrich-Freska found virus or antibody in the supernatants of the equivalence zone, thereby lending support to the evidence that the virus is acting as a single component. The latter authors also state that they are of the conviction that the virus-antiserum precipitate is insoluble in the zone of antibody excess (37).

In Figure 7, data published in the articles by Kleczkowski, and Schramm and Friedrich-Freska, obtained from the precipitin reaction in the zones of antibody excess and equivalence, are plotted with the corresponding data from antiserums, Nos. 94 and 96. The relationship between the proportions in which the virus and antibody are mixed (X-axis) and the ratio of antibody to virus in the precipitate (Y-axis) is shown. The

proportions in which the two reagents were brought together are calculated on the basis of the antibody content of the various antiserums as determined by the investigators themselves. In terms of milligrams protein per cc., the antibody amounts to 0.150 in antiserum No. 96, and 0.216 for antiserum No. 94, while Kleczkowski reports approximately 0.60, and Schramm and Friedrich-Freska, an antibody content of 4.95. Plotting the

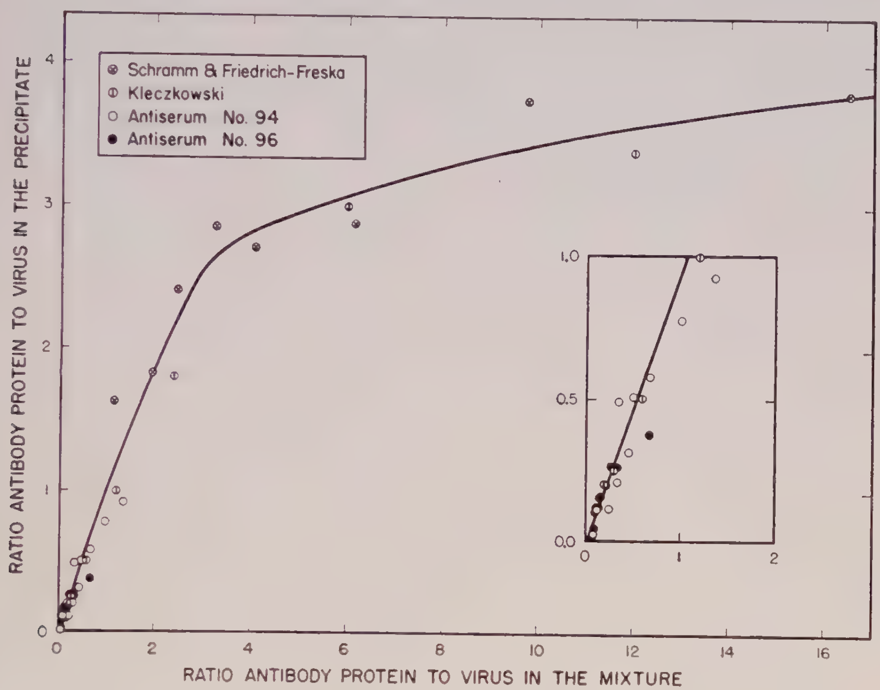

FIGURE 7. Comparison of data of other investigators of the tobacco virus-antiserum system with that presented in this article. Relationship between ratios of antibody protein to virus in the precipitate and in the mixture. Inset is enlargement of first part of curve which includes all data for antiserums, Nos. 94 and 96, and four experimental points of Kleczkowski.

data in this manner enables one to determine at a glance the reaction range covered by the various investigators using antiserums showing as much as a 33-fold difference in titer. The ratios of antibody to virus in the precipitate obtained for the three different sets of experiments from antibody excess through the equivalence zone varied from 0.94 to 0.02 for antiserums Nos. 94 and 96, to a range of 3.4 to 0.2 for Kleczkowski and 3.8 to 1.6 for Schramm and Friedrich-Freska corresponding to ratios of antibody to virus in the mixtures ranging from 1.4 to 0.1, 12.0 to 0.2, and 16.5 to 1.6, respectively. It will be noted that where antibody is present in great excess, the composition of the precipitate is relatively little affected

and the curve tends to flatten out. But, if comparisons of the ratios of antibody to virus in the precipitates are made at a number of different points where approximately the same proportions of the reagents were used, the composition of the precipitates from the different sets of experiments show fair agreement.

SUMMARY AND CONCLUSIONS

1. An outline is given of the development of the serum-precipitin reaction from its use primarily as a qualitative test to an accurate quantitative method. The successful application of the technic to both low- and high-molecular-weight antigens is cited.

2. The experimental results obtained from a study of the reaction with the high-molecular-weight antigen, tobacco-mosaic virus, are submitted to statistical analysis. A logarithmic presentation of the data is thus suggested which gives closer agreement between the calculated and observed values than that obtained by equations previously derived by other investigators, employing low-molecular-weight antigens.

3. The results obtained by others on the tobacco-mosaic virus-antiserum system are compared and found to be in general agreement with those herein described.

4. As in other immune systems when increasing amounts of virus are added to a constant amount of antibody: (a) the amount of precipitate increases successively, through the zones of antibody excess and equivalence and a part of antigen excess, and (b) the ratios of antibody to virus in the precipitate decrease successively in the zones of antibody excess through equivalence.

5. As in other immune systems the composition of the precipitate is a function of the proportions in which the antibody and virus are mixed.

6. In common with other high-molecular-weight antigens, the ratios of antibody to virus in the precipitate fall below unity near the region of equivalence.

7. The tobacco-mosaic virus reacts as a single component.

8. The quantitative serum-precipitin reaction is entirely applicable to the tobacco-mosaic virus-antibody system and should prove to be a valuable technic in the investigation of the nature of the virus and its related strains.

The authors wish to express their appreciation to Dr. Michael Heidelberger for a critical reading of the manuscript.

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WILL BUFFALO GRASS BE USEFUL IN EASTERN UNITED STATES?

William Crocker

In the late fall of 1941 the author brought back to Yonkers, New York, from Boyce Thompson Southwestern Arboretum, Superior, Arizona, a few plants of a strain of buffalo grass (*Buchloe dactyloides* [Nutt.] Engelm.). The plants were kept in pots in the greenhouse during the winter and in the early spring set out in well drained sandy loam soil (pH 6.1 to 6.8) with the plants spaced a foot apart in both directions. During the summer of 1942 the plants produced some runners that rooted but failed to cover the ground completely. They turned brown during the fall and winter and to superficial appearances were dead. During the summer of 1943, however, they produced many runners which covered the ground completely with a dense resilient tough sod. This sod was in excellent condition in the fall of 1944 after the original plants had grown during the two wet seasons (1942 and 1943) and the very dry season of 1944. In the spring of 1944 a portion of the plot that had been stripped for sodding a bank was reseeded by scattering shredded rhizomes over the ground and covering them with a thin layer of soil. By mid-summer the rhizomes had covered the ground completely with a thick sod which thrived during the very dry months of July, August, and September. Both the one- and three-year-old sods are shown in Figure 1.

In the spring of 1944 a bank with a southwest exposure that had been difficult to keep covered with the best mixtures of lawn grasses for the region was sodded with buffalo grass and allowed to grow during the very dry summer with one watering and with only one mowing in mid-June. Figure 2 shows a photograph of this bank taken on September 1, 1944. At the time the photograph was taken the grass covered the bank completely as a dense sod and had crept about six inches on the flat part of the lawn. The grass was gray-green, characteristic of this grass during the growing season. By October it had started to turn brown, and by November 15 it was entirely straw-brown.

These experiences indicate that buffalo grass will not only grow but will thrive and form dense sod in the humid east. It is apparently much more resistant to chinch bug attacks than the lawn grasses currently used in eastern United States. The photograph (Fig. 1) shows the bent grass badly attacked by chinch bugs whereas the adjoining buffalo grass shows no injury. It is also comparatively easy to keep free from crab grass by weeding. During the crab grass period of the growing season, the buffalo grass

is not mowed. This forces the crab grass to grow vertically mainly with a single stalk. In this condition it is easy to pull. The deep-rooting and drought-resistance of the buffalo grass enables it to thrive on lawns as well as on sun-exposed banks during dry seasons.

The Fort Hays branch of the Kansas Agricultural Experiment Station has made an extensive study of buffalo grass (2). Wenger speaks of the

FIGURE 1. A. At top, above upper black line, is a buffalo grass sod developed from rhizomes planted in the spring of 1944 and photographed September 1, 1944. Between black lines is a buffalo grass plot developed from individual plants set one foot apart in the spring of 1942 and photographed September 1, 1944. Below this is bent grass sod injured by chinch bugs. B shows a closer view of buffalo grass including both the 1-year and 3-year-old sods. The bare spots shown in these figures are regions where sods were removed for the purpose of getting separate plots of staminate and pistillate plants.

grass as "a low-growing, long-lived, drought-resistant perennial grass which spreads vegetatively by numerous surface runners. Profuse branching of the runners enables this grass to form a dense sod which is capable of withstanding considerable trampling." Wenger also says (2, p. 8): "Buffalo grass occurs naturally throughout the Great Plains region from the Canadian line to the Rio Grande river. It is of greatest importance in the Central Plains area where it and blue grama (*Bouteloua gracilis* Lag.) comprise more than 90 per cent of the native vegetation on the non-sandy soils. This area of major importance embraces approximately 190,000 square miles and includes south-central and the extreme western half of Nebraska, the western half of Kansas, the eastern fourth of Colorado, the western third of Oklahoma and northwest Texas."

FIGURE 2. An exposed dry bank sodded with buffalo grass in April 1944 and photographed September 1, 1944. This was mowed once in June but allowed to grow the rest of the season without mowing. The bank was watered once during the very dry summer. The soil below the sods bore many crab grass seeds. The crab grass plants were pulled in late July and late August.

A plant with as broad latitudinal distribution as this is likely to exist as many strains adapted to different climatic conditions. Indeed, Wenger speaks of the great variation in many respects. Amongst these is a taller strain that produces much more forage than the usual strains. The Fort Hays branch station is producing seeds of this strain in marketable amounts. This great variation means that in testing the grass in new regions, several strains should be tried with the hope of getting one well adapted to the particular region.

Until recently, seeding new areas had to be done by use of rhizomes or pieces of sods for seed was not available on the market in sufficient quantities. The high costs of vegetative sodding limited seeding to small areas or

to areas where high costs were justified such as lawns, golf courses, and athletic and air fields. The bulletin of the Fort Hays station (2) describes the development of methods by which it is now possible to put quick-growing seeds on the market in quantities at a reasonable price; machinery has been developed that will harvest the low-growing seed and even pick up the shattered seed from the ground and methods are now known for overcoming the long delay in germination of the seed by which the after-ripened seeds can be put on the market dry and ready to drill.

On the basis of the limited tests of buffalo grass in eastern United States, it seems to have its greatest promise for covering badly exposed dry banks on lawns and in road and railroad cuts. If, however, one is willing to endure a brown lawn during late fall, winter, and early spring in exchange for a dense resistant sod he may use it as a lawn grass. If the experience on the western plains holds in the east, the lawn and perhaps the banks would have to be replanted every six or eight years because of degeneration of old stands. The main reasons, however, for reporting on our very limited experience with buffalo grass at Yonkers is to lead others to test it in various regions of humid United States to find whether it will fill a need not already met by grasses presently grown in these regions. This experience with buffalo grass also suggests that other drought-resistant grasses of the Great Plains should be tested in the humid east. To this end this Institute is to start grass plots in 1945 of various strains of buffalo grass, three species each of *Bouteloua* and *Hilaria* along with the semi-tropical grass, "flawn" (*Zoysia matrella* Merr.) (1), which has received so much attention during the last few years as a deep-rooting drought-resistant grass which forms a dense tough sod capable of standing much traffic. "Flawn" is proving of value in eastern United States as far north as Connecticut on air fields and other locations where high temperature, drought- and traffic-resistance are required. The performance of "flawn" in this region as described in various popular publications seems similar to the performance of buffalo grass, as shown by our experience both as to undesirable and desirable characteristics. Both brown early in the fall and remain brown rather late in the spring; both are drought- and heat-resistant and form tough sods; and both seem to be relatively free from insect attack. From the very limited information to date, "flawn" seems to resist weed invasion better than buffalo grass. Certain strains of buffalo grass are hardy farther north than "flawn." Up to date "flawn" is propagated only vegetatively and buffalo grass can be grown from seeds as well.

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FURTHER STUDIES WITH DWARF SEEDLINGS OF NON-AFTER-RIPENED PEACH SEEDS

Florence Flemion and Elizabeth Waterbury

In previous reports it has been shown that seeds of peach (*Prunus persica* [L.] Stokes) and a number of other species which normally require a period at low temperature for germination to occur will produce plants without the low temperature stratification if they are placed under germination conditions after removal of all the seed coats. Such plants, however, are abnormal in appearance and growth and have been described as dwarfish (1, 2). The present paper reports results of some studies with these dwarfed seedlings as compared to plants from normally after-ripened seeds. Determination of relative amounts of root and top growth and grafting experiments have shown that the dwarf plants make ample root growth and the root system will support growth of normal tops, but dwarfed tops grafted on normal roots will retain their dwarfish characteristics. The results thus indicate that the seat of dwarfishness is in the top which makes poor growth apparently because of the lack of some essential constituent or perhaps because of the presence of an inhibitor. Removal of various portions of the storage material of normally after-ripened seeds, while seriously interfering with growth if large amounts were removed, did not result in growth habit similar to that of the dwarfs.

TECHNIQUE AND RESULTS

The normal fully after-ripened seedlings were obtained (3) from seeds which, after the hard outer coats had been removed, were mixed in moist peat moss and kept at 5° C. for two to three months prior to being planted. The dwarfed seedlings were obtained (2, 3) from non-after-ripened seeds which, after the outer and inner coats had been removed, were mixed in moist peat moss and held at room temperature. Within five to ten days the germinated seeds had developed adequate roots so that they could be planted. All of the seedlings were planted in three-inch pots containing soil and kept in the greenhouse maintained at approximately 70° F. during the winter months.

The hypocotyls of the non-after-ripened seeds develop almost as rapidly as the hypocotyls of fully after-ripened seeds. The subsequent behavior of the dwarfed in contrast to normal seedlings was observed by differences in size, dry weight determination, etc. Twenty-five each of after-ripened and non-after-ripened seedlings were planted in soil and placed in the greenhouse. After 90 days the average height of the normal seedlings was 390 millimeters while that of the dwarfs was only 35 mm. Several of the

FIGURE 1. Peach seedlings, planted March 30, 1940. Upper row, non-after-ripened dwarfed seedlings: A. intact plants, B. same as A with soil removed. Lower row, after-ripened normal seedlings: C. intact plants, D. same as C with soil removed. A and C photographed June 19, 1940; B and D photographed June 20, 1940. (× 0.143)

seedlings were photographed the following day, both before and after the soil was removed from the roots. As seen in Figure 1, the root system of the dwarf appears to be greater in proportion to top growth than is the root of the normal when compared with its shoot development.

In other experiments lots of fully and partially after-ripened as well as non-after-ripened seedlings were planted and kept in the greenhouse for several months. After height measurements were made, the tops were cut off, the soil was removed from the roots by washing in water, and the material was then spread out to dry at room temperature for subsequent dry weight determinations. The results are presented in Table I. There was considerable increase in both height and dry weight as the period of after-ripening progressed at 5° C. In regard to the ratio of root to top the roots represented a greater proportion of the total dry weight in the dwarfs than in the controls. Thus, the dwarfs seem to have ample roots not only from apparent size relationships but also in dry weight determinations.

TABLE I

HEIGHT AND DRY WEIGHT DETERMINATIONS OF NON-AFTER-RIPENED, PARTIALLY AFTER-RIPENED, AND FULLY AFTER-RIPENED PEACH SEEDLINGS

Days at 5° C. in moist peat moss prior to planting	Greenhouse plantings, date	Time in greenhouse, days	No. of plants	Type of growth	Average height, mm.	Average dry weight, g.	Total dry weight, %	
							Tops	Roots
0	Jan. 13, 1941	78	51	Dwarf	33	0.986	66	34
82			66	Normal	233	2.176	78	22
0	Jan. 8, 1941	127	23	Dwarf	32	2.028	54	46
13			14	Intermediate	70	2.842	52	48
41			23	Normal	238	3.898	58	42
55			28	Normal	265	3.968	65	35
69			17	Normal	249	4.031	67	33
83			18	Normal	217	4.280	68	32
0	Oct. 30, 1940	153	28	Dwarf	36	1.763	55	45
26			18	Intermediate	152	3.415	60	40
54			8	Normal	169	3.472	60	40
82			15	Normal	203	3.503	59	41

A series of grafting experiments was undertaken to determine whether the roots of these dwarf seedlings were capable of sustaining normal development and also to determine whether various parts of normal seedlings could induce normal development in the dwarfs. The material was chosen during the winter months when in the succulent early actively growing stage. The scions and stocks were held together until they united by wrapping with thread. The cleft and slant type grafts were equally successful. The plants were kept under bell jars and shaded with newspaper on sunny

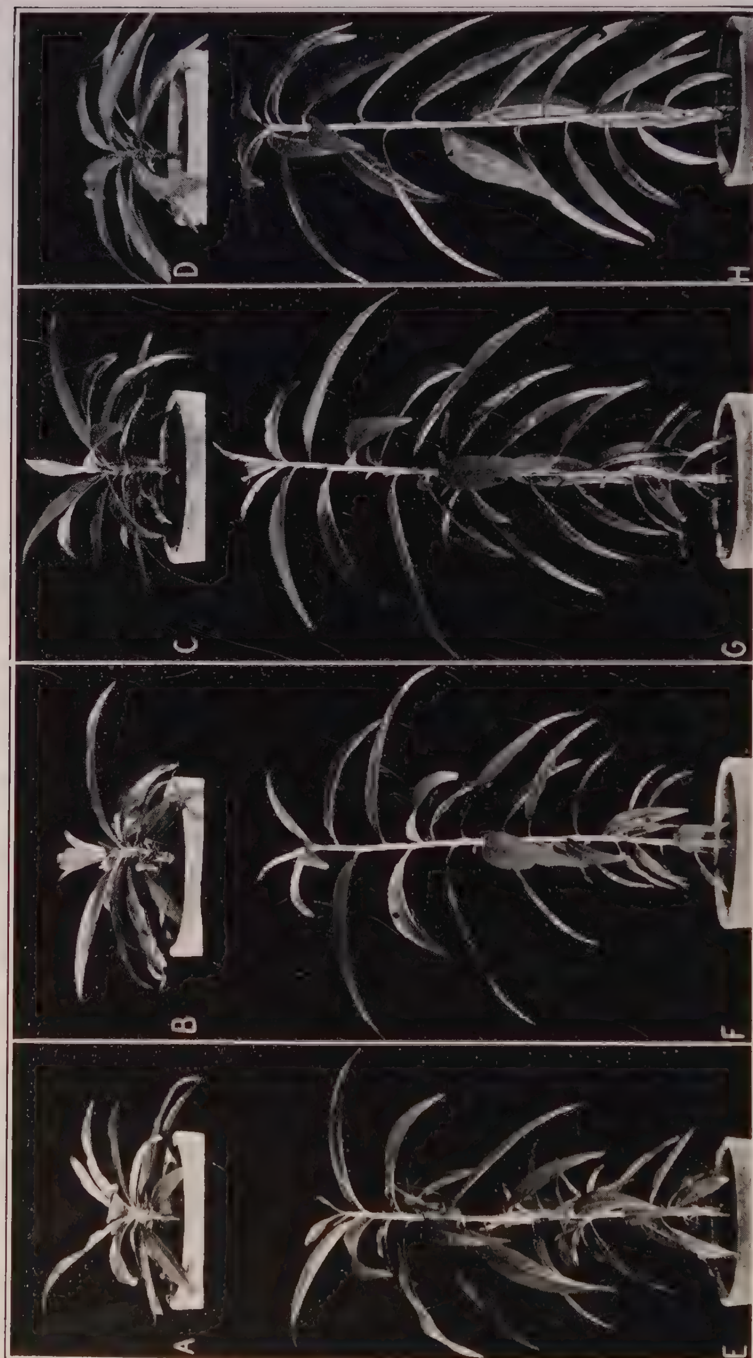

FIGURE 2. Peach seedlings (× 0.25). Upper row, dwarfed seedlings; lower row, normal seedlings. A. Dwarf control; B. dwarf tip grafted on dwarf understock; C. dwarf tip grafted on normal understock; D. dwarf seedling with a piece of stem from normal seedling interposed by grafting; E. normal control; F. normal shoot grafted on normal understock; G. normal shoot grafted on dwarf understock; H. normal seedling with a piece of stem from dwarf seedling interposed by grafting.

days. Within two to three weeks the string could be removed and the plants were held under the bell jars for a few additional days. Of 93 grafts made from December 16, 1940 to March 3, 1941, 77 united and remained healthy plants.

Parts of normal seedlings were grafted on the understock of dwarfed seedlings and vice versa. A dwarf and a normal control are illustrated in Figure 2 A and E, respectively. Subjecting the plants to the shock of grafting had no effect as shown in Figure 2 B and F, for these are illustrations of a dwarf plant where a dwarf scion was grafted on a dwarf seedling and a normal plant consisting of a normal scion grafted on a normal seedling.

When the understock of a normal seedling was used and the growing tip of a dwarfed seedling was grafted thereon (Fig. 2 C), the seedling remained dwarfed although the root system was that of a fully after-ripened seedling and was capable of maintaining normal growth. The growing tip of a normal seedling when grafted on the understock of a dwarfed seedling (Fig. 2 G) subsequently continued with its normal development. Thus, the understock of a dwarfed plant is adequate for maintaining normal development but the growing tip of a dwarf is unable to become normal when grafted on a normal seedling. The seat of this type of dormancy is in the growing top. Dwarfed plants were decapitated about seven days after planting and all the subsequent development from lateral buds was also dwarfish, thus illustrating that the dormancy is not localized in the terminal bud (Fig. 3).

When a piece of stem (5 to 15 mm.) from a normal plant was interposed by grafting in the stem of a dwarf, the plant remained dwarfed (Fig. 2 D). Likewise, when a piece of stem from a dwarfed plant was interposed in the stem of a normal plant, the plant remained normal (Fig. 2 H). In some plants when a dwarf tip had been grafted on a normal rootstock, a lateral bud on the normal stem below the union was permitted to develop as shown in Figure 4 A. Although this seedling had the root system of a normal plant and a lateral branch development on the normal part of the stem, the grafted dwarfed tip remained a dwarf. No substances from the normal parts stimulated the dwarfed top to develop into a normal plant. The effect of a possible stimulating substance in the normal plant or of any inhibiting substances in the dwarf plant was not in evidence in any of the above grafting experiments.

In addition, dwarfed and normal seedlings were planted so that their roots were in intimate proximity over a long period of time. As illustrated in Figure 4 B, a dwarf and a normal seedling were planted in the same pot and photographed after three and one-half months during which time there was no apparent influence exerted for each developed as is typical of its type.

Attempts were made to produce dwarfs from fully after-ripened seeds by removing various plant parts. In one experiment approximately one-half of the roots of both dwarfed and normal seedlings were removed about a month after planting. As far as the subsequent top growth of these plants was concerned, no effects were observed. After-ripened peach em-

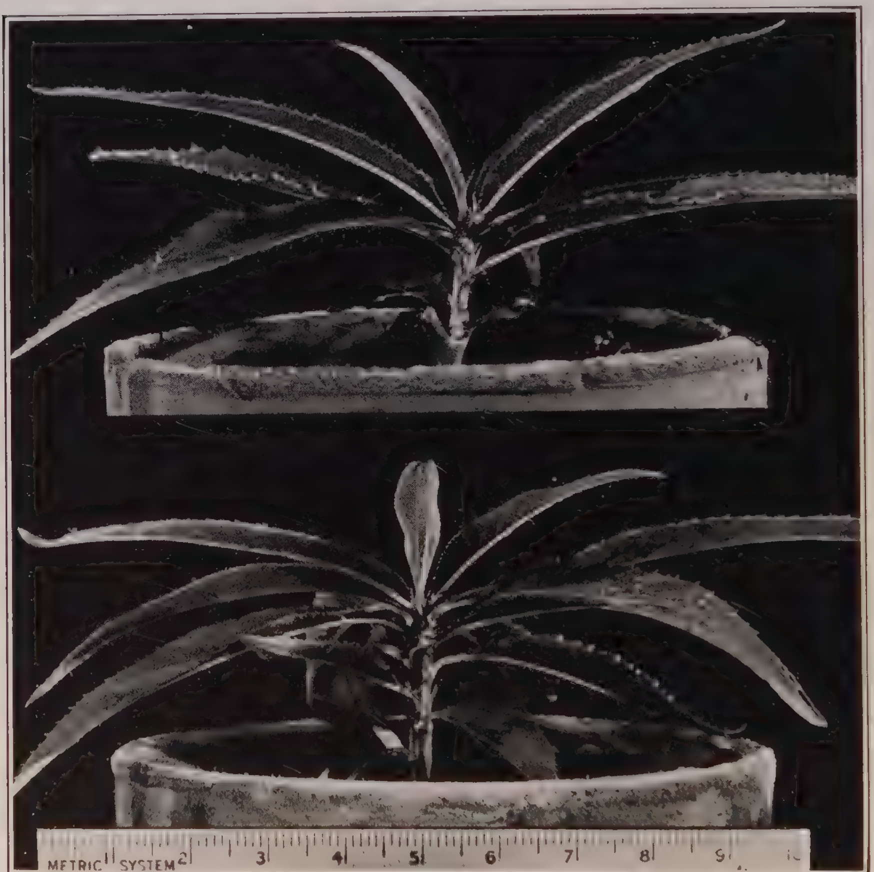

FIGURE 3. Non-after-ripened peach seedlings. Planted November 27, 1944. Upper plant, the growing tip (several millimeters) was removed on December 4, 1944, and a lateral bud subsequently developed; lower plant, control. Photographed, natural size, January 9, 1945.

bryos were deprived of all or parts of their storage material. Death resulted when both cotyledons were removed at the time of planting but when removed ten days later small but normal plants were obtained. When vari-

ous parts of the cotyledons were removed at time of planting, normal plants resulted except that, when approximately only one-third of one cotyledon remained, the plants were very small but otherwise normal. None of these miniature plants had the telescoping of internodes or other dwarfing characteristics so typical of seedlings obtained from non-after-ripened seeds.

That many profound changes occur during the process of after-ripening in seeds is again clearly illustrated by the results presented above. The

FIGURE 4. Peach seedlings (× 0.25). A. Dwarfed tip grafted on normal understock. The lateral branch is from a bud below the graft on the normal stem which was permitted to develop. Planted December 4, 1940, grafted December 16, 1940, photographed March 31, 1941. B. An after-ripened normal seedling (left) and a non-after-ripened seedling (right) planted in same pot on December 4, 1940 and photographed March 31, 1941.

breaking of seed dormancy in the peach by low temperature is in a sense a treatment for overcoming bud or vegetative dormancy since normal development is obtained by subjecting either the seed or the dwarfish seedling to low temperature.

SUMMARY

In grafting experiments with dwarf seedlings obtained from non-after-ripened seeds of peach, neither root nor shoot of normal seedlings induced

normal development in the dwarfs and conversely neither root nor shoot of dwarfs produced dwarfishness in the normal plants.

Various other attempts to produce dwarfs from after-ripened seeds were not successful. Even when the greater part of the storage material (cotyledons) was removed, the plants were normal but miniature and displayed none of the dwarfing characters.

Effect of stimulating substances from the normal plants or of inhibiting substances from the dwarfed plants was not apparent in any of these experiments.

The roots of dwarfed plants are not dormant and are capable of sustaining normal top development but the growing dwarfish shoot is unable to assume normal development either when on its own roots or when grafted on the understock of normal seedlings. Thus, the seat of dormancy in these non-after-ripened seedlings is in the shoot.

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A NOTE ON THE VIABILITY OF SEEDS OF MAGA, MONTEZUMA SPECIOSISSIMA

Lela V. Barton

INTRODUCTION

The rapid deterioration of maga seeds (*Montezuma speciosissima* Moc. & Sessé) after harvest coupled with the economic importance of the tree in yielding wood of good quality has made it desirable to determine conditions for keeping seeds viable over longer periods of time. Germination occurs shortly after the seeds fall from the tree, and may even start before the fall of the capsule. From this characteristic one would judge that desiccation might destroy the life of the embryo, which is large and occupies all of the space within the seed coat. Seed storage of maga was the subject of a study made by Marrero (2) and reported in 1942. The present experiments were performed with seeds from the same source but shipped to Yonkers, N. Y. before the tests were begun. The results are presented here with the idea of supplementing the data obtained by Marrero and of adding to the information necessary before a successful storage method can be evolved.

EXPERIMENTS AND DISCUSSION

The seeds were received in two shipments from Mr. L. R. Holdridge, Tropical Forest Experiment Station, Rio Piedras, Puerto Rico. The first lot which was shipped by air mail on May 16, 1940 and arrived in Yonkers, N. Y. on May 20, was contained in four small separate cellophane bags in a mailing tube. The seeds were still moist and apparently in good condition upon arrival. Out of 194 seeds examined, 82 were empty and 112 filled with embryos. There were brown spots on some of the cotyledons. After receipt the seeds were kept in the mailing tube at 5° C. overnight and then divided into two lots.

From the first lot with its original moisture content a germination sample was taken and the remainder were again divided into two sets and placed in tin cans with tight fitting lids sealed with sealing wax. One can was stored at 5° C. and the other at 20° C. The initial tests in moist granulated peat moss showed 40 per cent germination at a daily alternating temperature of 15° to 30° C., and 60 per cent germination at a constant temperature of 30° C. The latter method was used for subsequent viability tests of stored seeds, the first of which was made two weeks later. All of the moist seeds stored at 20° C. were dead at this time. Thirty per cent germination was obtained from those stored at 5° C. A further test made after one month of storage at 5° C. showed 20 per cent still viable. The seed sup-

ply was exhausted at this time. Only ten seeds were used for each germination sample in these tests since very few seeds were available.

The second lot of seeds was dried by spreading in the air in the laboratory for three days, after which germination tests revealed a complete loss of viability.

These preliminary tests indicated that maga seeds do not withstand much drying or temperatures as high as 20° C.

In 1941 a second larger shipment of seeds was received from the same source. These seeds had been collected early in the week of May 5 and extracted and taken to the Tropical Forest Experiment Station on May 7.

TABLE I

MOISTURE DETERMINATIONS AND VIABILITY TESTS OF MAGA SEEDS, 1941 CROP

Storage		Moisture content		% Germination after storage for months					
Moisture	Temp., °C.	% Wet wt.	% Dry wt.	0	0.5	1	3	6	9
Moist	−5				0	0	0	0	—
	5	33.1	49.6	66	68	50	20	0	—
	20				10	0	0	0	—
Dried 6 hrs.	−5				0	0	0	0	—
	5	21.1	26.7	68	32	28	0	0	—
	20				28	8	0	0	—
Dried 24 hrs.	−5				12	22	18	20	—
	5	10.4	11.6	30	38	30	20	24	14
	20				24	30	16	16	—
Dried 48 hrs.	−5				28	22	18	24	33
	5	8.3	9.1	16	22	24	8	14	24
	20				20	12	18	14	—

The seed lot, after sorting out the obviously bad seeds, weighed 1,380 grams. They were too wet to ship and were left on a table top to dry to a weight of 1,039 grams. They were then kept in a refrigerator until shipment on May 9. They reached Yonkers, N. Y. by air express on May 12. Thus the special storage tests were begun approximately one week after collection. Division of the shipment was made into four lots. One was stored as received, others after drying for 6, 24, or 48 hours at room temperature. Storage was in sealed containers at three different temperatures: −5°, 5°, and 20° C. Moisture determinations as well as germination tests in moist granulated peat moss at 30° C. were made at the time of storage, and germination tests were made after storage for 2 weeks, and 1, 3, 6, and 9 months Fifty seeds each were used for viability tests and seeds for each test were sealed separately in tin cans with tight fitting lids sealed with sealing wax.

Experimental results are shown in Table I. The moisture present in the seeds at the time of storage is expressed as percentages of both wet and dry weights of the seeds. When the seeds were received they contained 33.1 per cent moisture as calculated on the wet weight of the seeds. Spreading on blotters and drying in the laboratory for six hours reduced the moisture to 21.1 per cent. After further drying for 24 or 48 hours, 10.4 and 8.3 per cent moisture remained in the seeds.

While the seeds withstood drying for six hours under the conditions here employed without any loss in viability, an extension of the desiccation period to 24 hours reduced the germination capacity by one-half. A still greater injury from 48 hours of drying was indicated by the initial test after such treatment, since only 16 per cent germination was obtained. However, since later tests from all conditions showed great variability in results due in part at least to the small number of seeds available, it is doubtful whether this difference was significant.

The most striking storage effect shown in Table I is the extended viability of seeds with low moisture contents. It is surprising that reduction in moisture content to the point of injury to the seeds as evidenced by reduced germination power is still effective in maintaining viability for a comparatively long period. Even after nine months, at which time the seed supply was exhausted, viability of seeds containing 8.3 per cent moisture remained unimpaired at $-5°$ and $5°$ C. Drying to 10.4 per cent may have been equally effective. Furthermore, control of storage temperature was not important for these dried seeds within the limits of this experiment and up to six months of storage. Seeds with 21.1 and 31.1 per cent moisture deteriorated rapidly at $5°$ C., even more rapidly at $20°$ C., and could not survive a temperature below freezing for as long as two weeks.

Although Marrero (2) made no actual moisture determinations but rather dried seed lots with the aid of an electric fan to 87.5, 75, and 62.5 per cent of the original weight, his results are in general agreement with those obtained here from the two higher moisture contents used. He stored the seeds four days after collection, a factor also contributing to very high moisture contents.

It may be possible to dry the seeds in some way that will not impair their germination capacity. Grapefruit seeds which were injured by rapid drying on blotters in this laboratory to 52 per cent moisture were not injured at all by slow drying in a humid room at $5°$ C. to 18 per cent moisture (1).

SUMMARY

The life span of maga (*Montezuma speciosissima*) seeds is reduced significantly within two weeks under ordinary storage conditions. If the mois-

ture content is as high as 33 per cent of the wet weight, they may be kept for a month in sealed storage at 5° C. Drying at laboratory temperature at Yonkers, N. Y. to approximately 10 per cent moisture reduced the germination capacity by one-half but permitted retention of this germination capacity at $-5°$, 5°, or 20° C. for at least six months and possibly longer.

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VIABILITY OF SEEDS OF FRAXINUS AFTER STORAGE

Lela V. Barton

MATERIAL AND METHODS

Collections of fruits of *Fraxinus excelsior* L., made at Hilchenbach, Westfalen, Germany, in September 1934, and of *Fraxinus pennsylvanica* Marsh., made at the New York Botanical Garden, New York, N. Y., the same autumn, were used for storage tests to determine the life span of these seeds under certain controlled conditions.

Fraxinus seeds are among those which have been reported to lose viability quickly under ordinary storage conditions.

The structure of *Fraxinus* usually called a "seed" is really a dry winged fruit known as a samara. In these experiments the seeds were not removed from the fruits but rather the entire fruits were used both for storage and for germination. Usually one seed is produced per fruit but occasionally two or sometimes three seeds are found. Also twin embryos are known to exist within a single seed in this genus. The frequency of these abnormalities varies with the species and other conditions, but is not of sufficient extent to discredit germination percentages based on the number of fruits planted.

One of the most essential features of any storage test is the standardization of germination techniques so that each viability test is comparable to the ones made at earlier and later periods. *Fraxinus pennsylvanica* was found to germinate readily under a variety of conditions. However, a higher percentage of seedling production was secured after a winter in a cold frame following fall planting than after a winter in a greenhouse at 21° C. Plantings could be made in the cold frame with equal effectiveness in seedling stand and vigor any time between September and the following March in the region of Yonkers, N. Y. Results from a September and a March planting are shown in Figure 1. Fruits of *Fraxinus excelsior*, however, proved more specific in their germination requirements. Plantings made in September or October produced good seedling crops the following spring but if the sowing of the fruits was delayed until December or later a second winter was required before germination took place (Fig. 1). It seemed a matter of simple expediency, then, to adopt fall planting as a routine procedure for viability tests after various storage periods. The data presented below are based on seedling production in the early summer from fruits planted in September or October of the previous year and kept over winter in a cold frame with a board-cover.

The fruits were stored at two temperatures, laboratory and 5° C. Lots

were placed in open containers, in sealed glass flasks, and in desiccators over calcium oxide at each of these temperatures. Air-dry fruits were used for sealing in the glass flasks. Samples were removed for viability tests after 1, 2, 5, 6, 7, 8, and 9 years. Duplicates of 150 fruits each were used for the initial tests and after 1 and 2 years of storage. Later tests were made with duplicates of 75 fruits each.

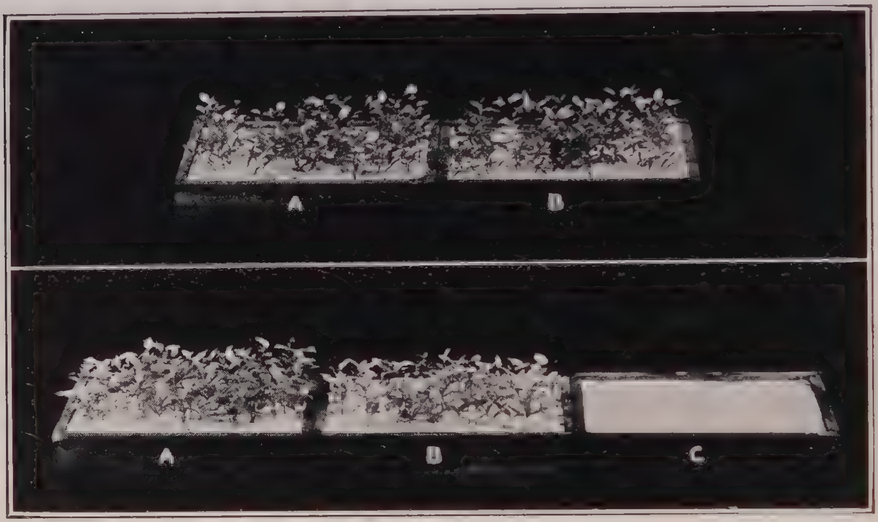

FIGURE 1. Top: *Fraxinus pennsylvanica*. Seedling production in the spring following planting in a board-covered cold frame in September (A), or March (B). 150 fruits planted in each flat. Bottom: *Fraxinus excelsior*. Seedling production in the spring following planting in a board-covered cold frame in September (A), October (B), and December (C). 150 fruits planted in each flat.

RESULTS AND DISCUSSION

Fraxinus pennsylvanica. From Table I it can be seen that 5° C. is superior to laboratory temperature for prolonging the life of seeds of this species. Sealed storage at 5° C. of seeds with about 8 per cent moisture permitted full retention of seedling-producing capacity for seven years. By the end of the eight-year period the viability was reduced by approximately 50 per cent and was lost completely after nine years of storage. Seeds with comparable moisture content stored in sealed containers in the laboratory had deteriorated significantly by the end of the two-year period and were without value after five years. It will be noted that seedling productions from seeds in sealed storage with approximately 7 per cent moisture in the laboratory as well as at 5° C. were somewhat higher (75 and 83 per cent) after one year of storage than the capacity as shown by the initial test (68

per cent). This is probably of no special significance but is due rather to the sample variation. Cutting tests of two lots of 100 fruits each revealed 13 and 16 fruits with empty seeds.

Open storage, whether in the laboratory or in a 5° C. room, was injurious to keeping quality. Definite reduction in the capacity to produce seedlings was evident after one year of storage and very few seeds survived for two years. The moisture content as well as the temperature fluctuated in the laboratory. The extent of the moisture fluctuation has been deter-

TABLE I

RESULTS OF TESTS MADE ON FRAXINUS FRUITS AFTER STORAGE UNDER VARIOUS CONDITIONS

Species and initial % seedling production	Storage conditions		% Moisture*	% Seedling production after years of storage					
				1	2	5	7	8	9
F. pennsylvanica 68	Laboratory	Open	3.9	36	2	0	—	—	—
		Sealed	6.8	75	45	2	—	—	—
		Over CaO	0.4	1	0	1	—	—	—
	5° C. room	Open	13.3	26	0	0	—	—	—
		Sealed	7.6	83	55	51	63	39	0
		Over CaO	2.3	65	49	36	45	34	—
F. excelsior 53	Laboratory	Open	8.9	42	24	1	—	—	—
		Sealed	10.3	16	0	0	—	—	—
		Over CaO	0.3	29	13	0	—	—	—
	5° C. room	Open	15.4	51	22	0	—	—	—
		Sealed	10.8	66	67	46	55	0	0
		Over CaO	2.4	43	40	16	1	0	—

* After one year of storage. Percentages based on dry weights of fruit lots used.

mined for different types of seeds during the course of the year in the laboratory at Yonkers, N. Y. (1). A maximum moisture content is reached in August, declining to low moistures which prevail from November to February. The moisture determinations as shown in Table I were made in September and probably represent intermediate values. At no time is the amount of water absorbed by seeds in the laboratory at Yonkers, N. Y. as great as that taken up in the 5° C. room in which the relative humidity is very high and fairly constant. *F. pennsylvanica* fruits, for example, contained 13.3 per cent moisture after a year of storage in open containers at 5° C. as opposed to 3.9 per cent after the same length of time in the laboratory.

Drying the fruits by storage in a desiccator over calcium oxide was injurious when the moisture content was reduced to 0.4 per cent as occurred at laboratory temperature. Such seeds did not survive one year of storage. Seeds stored in a similar way in the 5° C. room still contained 2.3 per cent

moisture. This might be accounted for by the smaller capacity of the desiccators used in the 5° C. room and the consequent smaller amount of calcium oxide. Moisture determinations made after two years of storage in desiccators indicated the maintenance of these same moisture contents. Seedlings produced from seeds kept under various storage conditions for two years are shown in Figure 2.

Thus, once again, it has been demonstrated that moisture content and temperature go hand in hand in the determination of the life span of seeds.

FIGURE 2. *Fraxinus pennsylvanica* seedlings produced after a winter in a board-covered frame following storage for two years in the laboratory (A), or in a 5° C. room (B). Left to right: open, air-dry sealed, over calcium oxide. 150 fruits planted in each flat.

Steinbauer (2), as a result of his experiments with *Fraxinus*, concluded that these seeds are much more sensitive to moisture content than to temperature. Together with other species of ash he stored seeds of *Fraxinus pennsylvanica* in sealed containers at 25° C. with controlled relative humidities which resulted in 1.68, 5.36, 6.49, and 9.85 per cent moisture content in the various seed lots. Tests made after a year showed 53, 48, 50, and 1 per cent viable respectively. He also stored air-dry seeds, containing 7.30 per cent moisture, in sealed containers at 5°, 20°, 25°, and 30° C., and found no differences after a year of storage (54, 49, 56, and 51 per cent). They tolerated drying down to 1.68 per cent without injury and kept equally well at 1.68 to 7.30 per cent moisture for a year; 9.85 per cent moisture caused deterioration so that only 1 per cent germinated after a year of storage. Data were not presented for longer periods of storage.

The results here reported are in agreement with those of Steinbauer when storage for one year only is considered. However, it is apparent that as the time in storage is increased the temperature plays a very important rôle in maintenance of viability—eight years at 5° C. as compared to two years under comparable moisture conditions in the laboratory.

Taylor (3) has studied the life span of seeds of *F. pennsylvanica lanceolata* and has reported a serious loss of viability by the end of one and one-half years' storage at warehouse temperatures.

Fraxinus excelsior. The favorable effect of storage at 5° C. over that at laboratory temperature for keeping quality for long periods was demonstrated (Table I). The somewhat shorter life span of seeds of this species as compared with seeds of *F. pennsylvanica* may have been due to the

FIGURE 3. *Fraxinus excelsior* seedlings produced after a winter in a board-covered frame following storage for two years under various conditions. Stored in the laboratory (A), or in a 5° C. room (B). Left to right: air-dry open, air-dry sealed, over calcium oxide. 150 fruits planted in each flat.

higher moisture content of the air-dry seeds at the time they were placed in sealed containers. It will be recalled that Steinbauer (2) found that with a moisture content as high as 9.85 per cent rapid deterioration occurred at 25° C. while 6.49 per cent was without a harmful effect on seeds of *F. pennsylvanica*. Thus it is seen here that seeds sealed with approximately 10 per cent moisture deteriorated rapidly in the laboratory but it should be pointed out that they kept fully viable for two and possibly five years and still retained about two-thirds of their seedling production capacity for seven years when stored at 5° C.

Open storage was better than sealed storage in the laboratory in the case of these seeds which had high moisture contents. Drying to approximately 2 per cent moisture reduced the germination of *F. excelsior* seeds and shortened their life span at 5° C.

Seedling production after two years of storage is shown in Figure 3.

SUMMARY

Seeds of *Fraxinus pennsylvanica* and *F. excelsior*, with moisture contents of approximately 7 and 10 per cent respectively, may be kept for at least seven years by using sealed containers at 5° C.

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DERIVATIVES OF PIPERIC ACID AND THEIR TOXICITIES TOWARD HOUSEFLIES

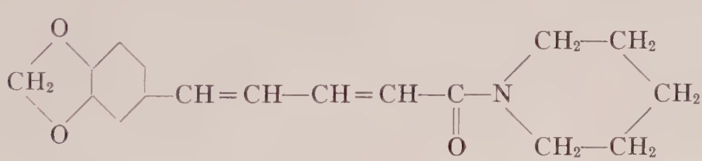
Martin E. Synerholm, Albert Hartzell, and John M. Arthur

An accelerated search for materials which may be used as pyrethrin substitutes has uncovered a number of interesting substances possessing varying degrees of toxicity toward insects.

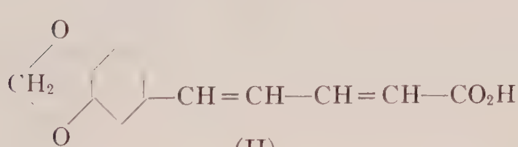
McIndoo and Sievers (14) in a summary of plants which have been used as insecticides include in their list black pepper (*Piper nigrum* L.). Hartzell (12) examined the acetone extract of the dried fruit of this plant for its toxicity, using mosquito larvae (*Culex quinquefasciatus* Say) as the test organism. The extract possessed exceptional toxicity, the LD_{50} being 29 p.p.m. based on the crude plant material.

Harvill, Hartzell, and Arthur (13) showed by the standard Peet-Grady procedure that piperine (I), the principal alkaloidal constituent of black pepper, was more toxic toward houseflies (*Musca domestica* L.) than pyrethrins. However, the paralyzing action expressed as knockdown (per cent of flies rendered unable to fly within ten minutes) was less than for sprays containing the same concentration of pyrethrins.

These properties of piperine made a study of related compounds seem worthwhile. Piperine may be easily converted to piperic acid (II) and it was decided to prepare a variety of amides and esters of this acid to be tested for their insecticidal action.



(I)



(II)

MATERIALS

Piperine. This material was generously supplied by S. B. Penick & Company of New York, N. Y.

Piperic acid (5). This acid was obtained in 90 per cent yield by re-

fluxing for six hours 200 g. of piperine in 1200 ml. of 20 per cent alcoholic potassium hydroxide. The mixture, after dilution with one liter of water, boiling with "Norite," and filtering was acidified. The crude acid after being dried, melted at 211° and was of sufficient purity for the preparation of the amides and esters. One recrystallization from alcohol produced fine yellow needles melting at 215°.

Amines and alcohols. These were obtained commercially with the exception of bis-β-chloroethylamine, piperonyl amine, and piperonyl alcohol.

Bis-β-chloroethylamine hydrochloride. This was prepared in good yield by the action of a slight excess of thionyl chloride on diethanolamine in dry benzene. The crude hydrochloride (m.p., 210°) was filtered and used as such.

Piperonyl amine (15). Piperonaldoxime was reduced to piperonyl amine by sodium amalgam in a glacial acetic acid—alcohol mixture. A solution of 30 grams of the oxime in 100 ml. of 95 per cent alcohol and 45 ml. of glacial acetic acid was shaken with 320 g. of 5 per cent sodium amalgam, while being cooled with tap water. When reduction was complete, the mixture was poured into enough water to dissolve the solids. The mercury was separated and the aqueous solution neutralized and finally saturated with potassium carbonate. The oily amine was extracted with benzene, dried with anhydrous potassium carbonate, and converted to the hydrochloride using dry hydrogen chloride gas. The yield of hydrochloride after filtering and drying was 25 g. (m.p. 225°).

Piperonyl alcohol. Piperonal was reduced with formaldehyde to the alcohol according to the directions in the literature (3) for the preparation of *p*-methyl benzyl alcohol.

Piperic acid chloride. The acid chloride was prepared in dry benzene by warming the acid with an excess of thionyl chloride. The excess of this reagent was removed on the steam bath and under diminished pressure. The crude acid chloride, which solidified to orange crystals, was not purified further but was taken up in fresh benzene and used as such in the preparation of the amides and esters.

Piperic acid amides. These were obtained in benzene solution either by warming with two equivalents of the amine or, as in the case of the more expensive amines, by the Schotten-Baumann reaction. The amides were easily separated from the benzene by the addition of petroleum ether. In nearly all of the cases reported here, they are white or cream colored solids. Their melting points and the analyses of those which have not previously been reported in the literature are given in Table I.

Piperic acid esters. These were all obtained, with the exception of β-thiocyanoethyl piperate, by warming for about one hour equivalent amounts of the appropriate alcohol with piperoyl chloride in benzene. The

esters solidified on removal of the solvent and cooling. They were recrystallized from alcohol or dilute alcohol to give low melting solids whose melting points are reported in Table II. Analyses for new compounds are also given. The β-thiocyanoethyl piperate was obtained from β-bromoethylpiperate by refluxing the latter with an excess of sodium thiocyanate in alcohol.

TABLE I

N-SUBSTITUTED PIPERIC ACID AMIDES

Piperic acid amide	M.p., °C. (uncorrected)	Nitrogen, % (Kjeldahl)*	
		Calcd.	Found
Unsubstituted piperamide	200	6.45	6.47
Methyl-	188–9	6.05	6.08
Ethyl-	164–5	5.71	5.76
n-Propyl-	162–3	5.40	5.42
Allyl-	149–50	5.45	5.51
n-Butyl-	151	5.12	5.12
iso-Butyl-	165–6	5.12	5.20
iso-Amyl-	144–5	4.87	4.97
Cyclohexyl-	188–9	4.68	4.66
2-Octyl-	144	4.26	4.22
Benzyl-	180–1	4.55	4.61
Piperonyl-	188–9	3.99	3.99
Phenyl-	198	4.77	4.82
2-Bromo-4-methylphenyl-	210–1	3.64	3.48
Dimethyl-	163–4	5.71	5.68
Diethyl-	94–5	4.92	4.89
Di-*n*-propyl-	64–5	4.65	4.83
Di-*iso*-propyl-	86–7	4.65	4.69
Di-allyl-	75–6	4.71	4.47
Di-*n*-butyl-	93	4.26	4.26
Di-sec-butyl-	Oil (not purified)	4.26	—
Di-*iso*-butyl-	79–80	4.26	4.11
Di-cyclohexyl-	138–40	3.67	3.76
Bis-β-chloroethyl-	192–3	4.09	4.21
Bis-hydroxyethyl-	157	4.58	4.56
N-Piperoyl morpholine	157–8	4.88	4.88
Piperine	128–9	—	—

* Analyses were made by Miss Elisabeth Heber-Smith.

"Deo-base" (purified kerosene fraction). This odorless petroleum fraction is supplied by insecticide manufacturers for use in making up fly sprays.

Official Test Insecticide (O.T.I.) (16). This standard consists of a 0.1 per cent solution of pyrethrins in deodorized kerosene ("Deo-base") and was supplied by the National Association of Insecticide and Disinfectant Manufacturers, Inc., New York, N. Y.

METHOD OF TESTING

Solutions of the compounds to be tested were prepared by dissolving them in a small amount of alcohol or acetone (usually 10 ml.) and adding "Deo-base" to make 50 ml. of solution. Whenever tests were run using

pyrethrins to ensure a high knockdown, these were added to the solutions as a kerosene extract in an amount necessary to give a final concentration of 0.025 mg. total pyrethrins per 100 ml. of solution.

The solutions were tested in the large Peet-Grady chamber according to the standard procedure as described on pages 177-183 of "Blue Book" (18). The kills and knockdown values obtained were compared with those

TABLE II

PIPERIC ACID ESTERS

Ester	M.p., °C. (uncorrected)	Carbon, % Calcd.	Carbon, % Found	Hydrogen, %* Calcd.	Hydrogen, %* Found
Methyl-	146 (lit. 146)				
Ethyl-	78 (lit. 78)				
n-Propyl-	97	69.3	69.1	6.17	6.18
iso-Propyl-	53-4	69.3	69.6	6.17	6.18
Allyl-	51-2	69.7	69.5	5.45	5.42
n-Butyl-	Oil	(Not purified)			
n-Amyl-	57	70.8	71.0	7.00	7.11
n-Heptyl-	51	72.2	71.8	7.65	7.87
n-Decyl-	51	73.7	73.0	8.42	8.62
n-Dodecyl-	63	74.5	74.6	8.90	8.96
n-Tetradecyl-	64-6	75.4	75.2	9.23	9.43
Cyclohexyl-	85-6	72.1	70.9	6.23	6.63
Tetrahydrofurfuryl-	70-1	67.5	67.4	5.99	6.26
Benzyl-	78	74.1	74.2	5.22	5.09
Piperonyl-	97-102	68.2	67.9	4.56	4.69
Bornyl-	103-5	74.6	74.6	7.39	7.40
Phenyl-	Gum	(Not purified)			
o-Phenylphenyl-	149	77.9	77.9	4.88	4.87
o-Cyclohexylphenyl-	142	76.7	76.1	6.42	6.40
		Chlorine, % (Parr bomb) Calcd.		Found	
β-Chloroethyl-	96-7	12.6		12.8	
o-Chlorophenyl-	140	10.8		11.0	
2,4-Dichlorophenyl-	158-9	19.5		19.5	
p-Chlorothymyl-	141-2	9.22		8.95	
β-Diethylaminoethyl hydrochloride-	187-8	10.0		10.1	
		Sulphur, % (Parr bomb)			
β-Thiocyanoethyl-	105-6.5	10.6		10.6	
p-Thiocyanothymyl-	110	7.86		8.01	

* Analyses were made by Miss Elisabeth Heber-Smith.

obtained with the O.T.I. run on the same day. The results of tests made using 0.025 mg. of pyrethrins per 100 ml. of solution of the piperic acid derivative are shown in Tables III and V.

In order to study the toxicities of some of these compounds alone, tests were run in the absence of pyrethrins. The knockdown values were high

enough to enable the operators to follow the Peet-Grady procedure. The results are reported in Tables IV and VI.

Because the culture of flies with the proper resistance is contingent on rather narrow limits of temperature and humidity not always obtainable and as flies were needed for other testing purposes, it was not always possible to run several tests on each concentration. The data in Tables III, IV, V, and VI were taken from the runs in which the O.T.I. kills were closest to the desired 50 per cent.

TABLE III

TOXICITIES OF PYRETHRIN—PIPERIC ACID AMIDE MIXTURES TOWARD HOUSEFLIES. CONCENTRATION OF PYRETHRINS: 0.025 G. PER 100 ML. OF SOLUTION

Piperic acid amide	Grams per 100 ml. of solution	Kill, % after 24 hrs.	O.T.I. kill, %
Unsubstituted piperamide	0.1	45	57
Methyl-	0.1	47	57
Ethyl-	0.1	47	39
n-Propyl-	0.1	36	55
Allyl-	0.1	75	50
n-Butyl-	0.1	88	44
iso-Butyl-	0.1	61	55
iso-Amyl-	0.1	93	44
Cyclohexyl-	0.125	88	51
2-Octyl-	0.1	38	55
Benzyl-	0.1	77	43
Piperonyl-	0.3	55	64
Phenyl-	0.1	46	72
2-Bromo-4-methylphenyl-	0.1	59	58
Dimethyl-	0.1	36	40
Diethyl-	0.1	77	50
Di-*n*-propyl-	0.125	63	47
Di-*iso*-propyl	0.1	91	71
Di-allyl-	0.1	92	47
Di-*n*-butyl-	0.1	71	56
Di-sec-butyl-	0.1	80	52
Di-*iso*-butyl-	0.1	77	38
Dicyclohexyl-	0.1	49	50
Bis-β-chloroethyl-	0.5	30	29
Bis-hydroxyethyl-	0.1	17	40
N-piperoyl morpholine	0.1	26	48
Piperine	0.1	91	58
Piperic acid	0.2	24	47
Pyrethrins alone	0.025	19	48

Note: The knockdown was almost complete in every case.

DISCUSSION

In the work reported here, it has been shown that a wide variety of amides and esters of piperic acid are toxic toward houseflies.

Examination of Table III shows that the amides which are most toxic are those derived from primary or secondary alkyl or cycloalkyl amines, the

alkyl groups of which contain from three to seven carbons. Comparison of these data with those of Table IV brings out the synergistic action of the amides when used with pyrethrins—a property shared by a number of other substances, notably N-iso-butylundecylenamide (1, 2, 19), sesamin (2, 4, 10, 11, 17), N-iso-butyl 3,4-methylenedioxycinnamamide (fagaramide) (9), and the N-substituted amides of 3,4-methylenedioxybenzoic acid (piperonylic acid) (6, 7, 8).

TABLE IV

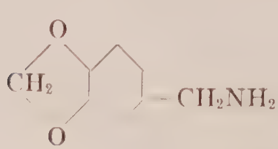
TOXICITIES OF PIPERIC ACID AMIDES TOWARD HOUSEFLIES

Piperic acid amide	Grams per 100 ml. of solution	Knockdown, % after 10 min.*	Kill, % after 24 hrs.	O.T.I. kill, %
n-Butyl-	0.5	79	36	55
	0.25	78	34	55
Cyclohexyl-	0.25	70	21	57
	0.125	73	46	57
Diethyl-	0.1	98	63	51
Di-*n*-propyl-	0.1	91	24	47
Di-*iso*-propyl-	0.1	95	50	71
Di-*iso*-butyl	0.1	91	31	38
Piperine	0.2	93	88	62
	0.1	89	33	57

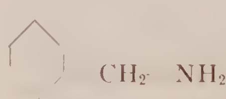
* The knockdown of the O.T.I. was almost complete in every case.

The amides of piperic acid derived from aromatic amines are relatively non-toxic as are the amides in whose alkyl groups a beta hydrogen has been replaced by a halogen or an oxygen atom as in bis-β-chloroethylpiperamide, bis-β-hydroxyethylpiperamide, and N-piperoyl morpholine.

The methylenedioxy group, which appears to be important in ascribing toxicity to the piperic acid residue (13) does not enhance the activity when it appears also in the substituent on the amide nitrogen. On the contrary, the amide derived from piperonylamine (III) is much less toxic than that prepared from benzylamine (IV).



(III)



(IV)

Tables V and VI clearly show that the most toxic esters of piperic acid are those derived from aliphatic or alicyclic alcohols containing more than three and less than seven carbon atoms. The phenyl esters are relatively non-toxic. The presence of halogens in the alcohol part of the molecule de-

TABLE V

TOXICITIES OF PYRETHRIN—PIPERIC ACID ESTER MIXTURES TOWARD HOUSEFLIES. CONCENTRATION OF PYRETHRINS: 0.025 G. PER 100 ML. OF SOLUTION

Ester	Grams per 100 ml. of solution	Kill, % after 24 hrs.	O.T.I. kill, %
Methyl-	0.5	35	36
Ethyl-	0.5	91	50
	0.25	68	46
n-Propyl-	0.5	86	50
	0.25	91	58
	0.125	77	56
iso-Propyl-	0.5	100	58
	0.25	97	59
	0.125	96	59
Allyl-	0.5	91	49
	0.25	63	48
n-Butyl-	0.5	98	68
	0.25	94	68
	0.125	90	68
n-Amyl-	0.5	73	50
	0.25	71	47
n-Heptyl-	0.5	69	55
	0.25	38	55
n-Decyl-	0.5	33	68
n-Dodecyl-	0.5	27	32
n-Tetradecyl-	0.5	37	59
Cyclohexyl-	0.5	98	59
Tetrahydrofurfuryl-	0.5	83	48
Benzyl-	0.5	95	58
	0.25	82	56
Piperonyl-	0.5	52	32
Bornyl-	0.5	79	47
β-Chloroethyl-	0.5	55	48
β-Bromoethyl-	0.5	87	56
β-Thiocyanoethyl-	0.5	47	56
β-Diethylaminoethyl-	0.5	65	32
Phenyl-	0.1	29	72
o-Chlorophenyl-	0.5	36	53
2,4-Dichlorophenyl-	0.25	23	52
2,4,6-Trichlorophenyl-	0.5	62	52
o-Cyclohexylphenyl-	0.5	58	70
o-Phenylphenyl-	0.5	35	59
p-Thiocyanothymyl-	0.5	40	48

Note: The knockdowns were almost complete in every case.

tracts from its effectiveness as an insecticide (compare ethyl piperate with β-chloroethyl piperate).

A synergistic action is noted in the use of the esters with pyrethrins. An outstanding example is the case of tetrahydrofurfuryl piperate.

TABLE VI

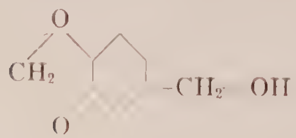
TOXICITIES OF PIPERIC ACID ESTERS TOWARD HOUSEFLIES

Ester	Grams per 100 ml. of solution	Knockdown, % after 10 min.*	Kill, % after 24 hrs.	O.T.I. kill, %
Methyl-	0.5	0	0	50
	0.25	0	0	50
Ethyl-	0.5	90	37	52
	0.25	90	28	52
	0.125	79	28	51
n-Propyl-	0.5	94	45	52
	0.25	94	50	52
	0.125	85	31	51
n-Butyl	0.125	91	22	51
n-Amyl-	0.5	95	50	48
	0.25	90	39	48
n-Heptyl-	0.5	82	25	50
	0.25	53	17	50
Tetrahydrofurfuryl-	0.5	98	35	52
	0.25	99	28	52
	0.125	82	31	51
β-Chloroethyl-	0.5	86	17	52
	0.25	89	34	52
	0.125	90	39	51
o-Chlorophenyl-	0.5	45	12	48

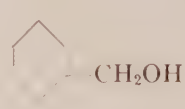
* The knockdown was almost complete in every case.

The presence of a thiocyano group in the alcohol part of the molecule renders the compound less effective. This is especially interesting in view of the fact that some of the most effective commercial insecticides are thiocyanates.

The ester derived from piperonyl alcohol (V) is less effective than that from benzyl alcohol (VI), affording an example in the ester series of the failure of an additional methylenedioxy group to increase the toxicity.



(V)



(VI)

The free piperic acid is non-toxic, a fact which is in agreement with expectations. In the chemistry of drugs it is well known that whereas derivatives of carboxylic acids may have pronounced physiological effects, the free acids are devoid of activity.

CONCLUSIONS

1. A wide variety of esters and substituted amides of piperic acid are toxic toward houseflies.

2. The most toxic amides are those derived from primary or secondary alkyl amines containing from three to seven carbon atoms. In the ester series the most effective are derived from alcohols with more than three but less than seven carbon atoms.

3. The amides and esters of piperic acid have a synergistic action when used in conjunction with pyrethrins against houseflies.

4. Esters prepared from phenols and amides from aromatic amines are relatively non-toxic.

5. The methylenedioxy group, which appears to be important in ascribing toxicity to the piperic acid residue, does not enhance the activity when it appears also in the substituents.

6. The presence of a thiocyano group in either an aliphatic or aryl piperate renders the compound less effective.

7. The presence of halogens does not seem to improve the activity. In some cases, namely the esters, their presence appears to impair the effectiveness.

8. Piperic acid itself is devoid of insecticidal activity.

9. Twenty-five previously unreported amides of piperic acid and 22 new piperic acid esters are described with their analyses.

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HISTOLOGICAL EFFECTS OF CERTAIN SPRAYS AND ACTIVATORS ON THE NERVES AND MUSCLES OF THE HOUSEFLY

ALBERT HARTZELL[1]

Activation is one of the most interesting phenomena in the study of the action of insecticides. An activator may be defined as a substance that renders another substance more effective, for example, in this case, one that increases the toxicity per unit weight. The use of activators has materially extended the supply of pyrethrum by making it possible to employ lower concentrations of the material in combination with less scarce and relatively non-toxic materials and still obtain the same toxicity as when pyrethrum is used at normal strengths alone. The literature on the mode of action of insecticides has been reviewed recently by Weed (14).

The present investigation is an attempt to determine the effects of certain fly sprays and activators on the nerve and muscle tissues of the housefly (*Musca domestica* L.) as differentiated by histological stains. The effects of the poison and activator on the nerve and muscle tissues in several instances were studied independently, and in another series the combined effects of the poison and activator were determined. It was thought that activation might possibly produce an effect on one or more tissue components of the cells.

As the activators when used alone at low concentrations produced little or no effect on the tissues, higher concentrations than those normally employed with the poison in fly sprays were used. In the case of rotenone a series of concentrations were used to study the knockdown effect (per cent of flies rendered unable to fly within ten minutes) of high concentrations as contrasted with no appreciable knockdown at low concentrations, in spite of the fact that such relatively low concentrations were lethal to houseflies.

The histological effects of pyrethrum and N-isobutylundecyleneamide on the central nervous system and muscles of the housefly have been described previously (3). A widespread clumping effect of the chromatin of the nuclei was noted with pyrethrum, while the activator N-isobutylundecyleneamide caused chromatolysis or dissolution of the chromatin.

Wigglesworth (15) has reported also the destructive action of pyrethrum on the central nervous system of insects. *Rhodnius prolixus* bugs that had been paralyzed for ten days by the application of pyrethrum in liquid paraffin to the antenna showed degeneration in the fused abdominal

[1] With the technical assistance of Bette Rubin, Medical Technician.

ganglia when the tissue was fixed in Carnoy's solution and stained with hematoxylin.

Recently Hartzell and Strong (4) have shown that piperine causes destruction of the fiber tracts and vacuolation of the nerve tissue of the brain of the housefly in preparations fixed in Dietrich's solution and stained with Bodian's method.

MATERIALS AND METHODS

Adult houseflies, five days old, of both sexes were sprayed under regular Peet-Grady test (12) conditions with various concentrations of the poisons and activators in a highly refined kerosene ("Deo-base"). Moribund individuals which were capable of slight movement on probing, but unable to fly, were collected at intervals of 10, 20, and 60 minutes, and 24 hours after spraying. Immediately after collecting, the abdomens were punctured with a needle, and the tissues were killed and fixed in 10 per cent formalin solution either overnight (16 hrs.) or for 24 hours. The flies were dehydrated whole or decapitated by means of a razor blade and the heads dehydrated in 95 per cent alcohol for two hours, then in absolute alcohol for two hours, and in chloroform for one hour, in chloroform and paraffin overnight (16 hrs.), and in paraffin bath I for one hour, and paraffin bath II for one hour at 60° to 62° C. m.p. Sections were cut from 4μ to 10μ in thickness and series were stained with hematoxylin and eosin-y (10), Bodian's silver albumose and gold chloride method (1), and toluidine blue (10). Controls were subjected to the same methods for comparison of the normal histology.

Compounds examined. Pyrethrum resin was dissolved in a highly refined kerosene ("Deo-base") to give a concentration of total pyrethrins of 1 mg. per cc., or approximately 0.1 per cent. Among the activators of pyrethrum sesame oil, piperine (6), and D.H.S. Activator were used in this study.

Sesame oil (U.S.P.) was used alone and in combination with pyrethrum extract.

Piperine was purified and recrystallized in the chemical laboratory of this Institute and was also used alone and in combination with pyrethrum.

D.H.S. Activator and Thanite were obtained from Hercules Powder Co., Wilmington, Delaware. The active ingredient of the first named activator is ethylene glycol ether of pinene, while the active ingredient of Thanite is a thiocyanoacetate of a secondary terpene alcohol.

Lethane 384 was obtained from Röhm and Haas Co., Bristol, Pennsylvania. The active ingredient as stated on the label is β-butoxy-β'-thiocyanodiethyl ether.

Pure crystalline rotenone was used in making up spray preparations in this study.

DDT or 2,2 bis-(p-chlorophenyl)-1,1,1-trichlorethane was prepared in the chemical laboratory of this Institute.

RESULTS

Hematoxylin and eosin-y method was found to be a satisfactory general stain for both insect nerves and muscles but did not differentiate nerve fibers as well visually as Bodian's method. Toluidine blue was found to be inferior to the first two named methods. Because of the great variations in dosage, times of exposure, and different methods of staining used in this study, the significant histopathologic changes are given in tabular form (Table I). The tissues examined were mainly the brain, the fused thoracic ganglia, and muscles particularly from a pair of striated muscles that run from the occiput to the base of the proboscis on either side of the caudal part of the brain. (For reference to all figures see Table I.)

EFFECTS ON NERVE TISSUE

The principal histopathologic changes produced by fly sprays and activators reported in this study are of three types: 1) dissolution of the nerve fiber tracts; 2) dissolution of cell components other than the fibers resulting in the prominence of nerve fibers; 3) vacuolation of the larger nerve cells. Pyrethrum poisoning belongs to the first type but in addition to the dissolution of nerve fibers the tissue is interrupted by clear spaces. DDT also causes partial dissolution of the nerve fibers, but the effect is not so pronounced as with pyrethrum.

In contrast to the first type, piperine, Lethane 384, and D.H.S. Activator appear to destroy the cellular components other than the nerve fibers leaving the fibers essentially intact. Lethane 384 and D.H.S. Activator in addition show deep-staining nuclei which are absent or less prominent with piperine. The third type of histopathologic change is illustrated by Thanite and sesame oil when applied at relatively high concentrations. Both produce vacuolation of the larger nerve cells.

When piperine and pyrethrum are combined, the effect is that of a partial dissolution of the nerve fibers plus a dissolution of certain of the cellular components. When sesame oil and pyrethrum are combined the nerve fibers are not only destroyed but the larger nerve cells are highly vacuolated. This is especially pronounced in tissues of flies that were placed in fixatives one hour after treatment.

Sprays containing relatively low concentrations of rotenone are lethal to flies although no knockdown is obtained. By increasing the concentration of rotenone it is possible to cause knockdown also. Microscopic examinations were made of tissues of flies that had been sprayed with various lethal concentrations and collected at intervals of 10 minutes, 60 minutes, and 24 hours after spraying. It was found that a spray containing 0.00625 per cent rotenone was lethal without producing knockdown, but microscopic examination of fixed and stained tissues showed no lesions. Flies

TABLE I

HISTOPATHOLOGICAL CHANGES PRODUCED IN BRAIN AND MUSCLE TISSUES OF THE HOUSEFLY BY INSECTICIDAL SPRAYS AND ACTIVATORS ALONE AND IN COMBINATION

Fig. No.	Treatment	Concn., %	Duration, min.	Significant histopathological changes
		A. Brain tissue stained with hematoxylin and eosin-y		
1A	Check	—	—	Normal tissue
1B	Pyrethrins	0.1	10	Dissolution of the fiber tracts and presence of clear spaces
1C	Piperine	0.5	10	Prominence of nerve fibers with almost complete dissolution of other cell components
1D	Piperine+ Pyrethrins	0.05 0.01	10	Dissolution of nerve fiber tracts and partial dissolution of other cell components
1E	Sesame oil	50.0	10	Vacuolation around large nerve cells
1F	Sesame oil+ Pyrethrins	10.0 0.05	60	Vacuolation and almost complete lysis of the tissue
2A	DDT	0.2	10	Partial dissolution of the fiber tracts; degeneration of nuclei
2B	Lethane 384	3.0	10	Prominence of nerve fibers and deep-staining nuclei
2C	Thanite	3.0	10	Vacuolation around large nerve cells
2D	D.H.S. Activator	0.5	10	Prominence of nerve fibers and deep-staining nuclei
2E	Rotenone	0.025	10	Partial dissolution of fiber tracts
2F	Rotenone	0.025	60	Vacuolation and disintegration of nerve tissue
		B. Brain tissue stained by Bodian's method		
3A	Check	—	—	Normal tissue
3B	Pyrethrins	0.1	10	Dissolution of fiber tracts and presence of clear spaces
3C	DDT	0.2	10	Fraying and dissolution of nerve fibers
3D	DDT	0.2	60	Vacuolation around nerve cells and fraying of nerve fibers
3E	Rotenone	0.025	10	Dissolution of nerve tracts and vacuolation
3F	Rotenone	0.00625	10	Little or no cellular degeneration
		C. Muscle tissue stained with hematoxylin and eosin-y		
4A	Check	—	—	Normal tissue
4B	Pyrethrins	0.12	10	Nuclei clumped into rod-like dense masses, and tissue occasionally fenestrated
4C	Piperine	0.5	10	Slight accentuation of Krause's membrane
4D	Piperine+ Pyrethrins	0.05 0.01	10	Nuclei clumped into rod-like dense masses
4E	Sesame oil	50.0	60	Nodes and Krause's membrane greatly accentuated
4F	Sesame oil+ Pyrethrins	10.0 0.05	10	Nuclei clumped and prominence of bands
4G	DDT	0.2	10	Nuclei clumped into rod-like masses
4H	D.H.S. Activator	0.5	10	Nodes and Krause's membrane greatly accentuated
4J	Thanite	3.0	60	Little or no change
4K	Lethane 384	3.0	10	Fraying of muscle strands and destruction of nuclear membranes
4L	Rotenone	0.05	10	Little or no change
4M	Rotenone	0.025	10	Little or no change

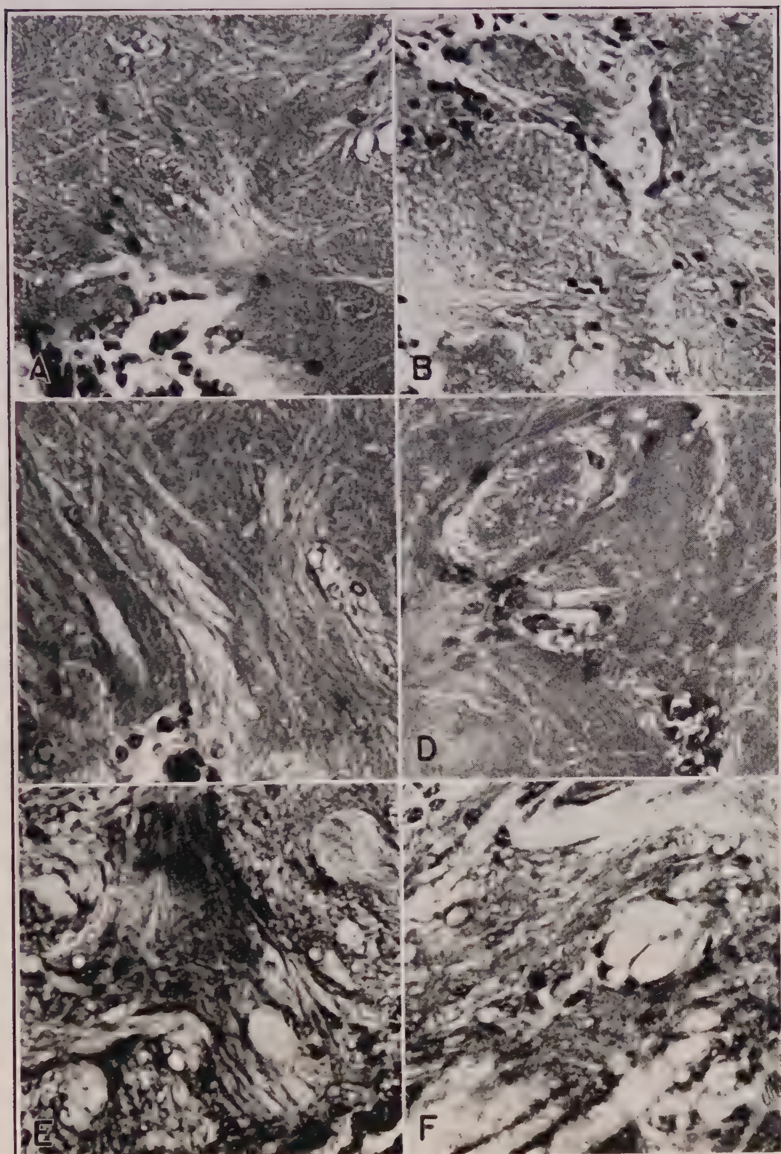

FIGURE 1. Sections through brain of housefly in region of corpus centrale and fiber tracts laterad of it, fixed in formalin and stained with hematoxylin and eosin-y (×500). B to E, 10 min.; F, 1 hr. after spraying. A. Check. B. Effect of pyrethrum, dissolution of fiber tracts and presence of clear spaces. C. Effect of piperine, prominence of nerve fibers. D. Effect of piperine+pyrethrum, dissolution of fibers and other cell components. E. Effect of sesame oil, vacuolation of nerve cells. F. Effect of sesame oil and pyrethrum, vacuolation and lysis of tissue.

sprayed with a higher concentration of rotenone (0.025 per cent) produced a knockdown of 94.7 per cent in ten minutes and a kill of 34.0 per cent in 24 hours. Histopathologic changes of the tissues of these flies included dissolution of the fiber tracts of the brain and vacuolation of the larger nerve cells in moribund flies that were collected ten minutes after spraying. Vacuolation was more pronounced in flies collected one hour after spraying. In addition to hematoxylin and eosin-y stain and Bodian's gold chloride and silver albumose method, brain tissue was stained with toluidine blue. The tissue of flies that had been rendered moribund by a 0.1 per cent pyrethrin spray showed typical tigrolysis. Nerve tissue of flies that had been sprayed by 0.5 per cent piperine in "Deo-base" exhibited the nerve fibers prominently. When piperine (0.05 per cent) was combined with pyrethrins (0.01 per cent) there was dissolution of the nerve fibers and trigrolysis.

Sesame oil at high concentrations (50 per cent) caused little or no pathologic change. When sesame oil and pyrethrum were combined at usual strengths for fly sprays, there was marked dissolution of the fiber tracts and the larger nerve cells were vacuolated.

EFFECTS ON MUSCLE TISSUE

The principal effects of the fly sprays and activators used in this study were of three types: 1) clumping of the chromatin of the nuclei; 2) nodes and Krause's membrane accentuated; 3) destruction of the nuclear membrane. The clumping of the nuclei in rod-like dense masses is characteristic of pyrethrum poisoning, to a lesser extent this is also true of DDT. Sesame oil and D.H.S. Activator when used at high concentrations showed the muscle nodes and Krause's membrane greatly accentuated, and piperine to a lesser degree. It is possible that differences in refractive index may be responsible for this effect. Lethane 384 caused a third type of pathologic change as exhibited by destruction of the nuclear membrane.

DISCUSSION

There are two points of view in regard to nerve lesions. 1. Nerve lesions are secondary and may possibly be induced by a fall in the respiratory activity of the central nervous system. McClure *et al.* (9) have demonstrated in higher animals the existence of anoxia in the central nervous system during anaesthesia brought about by narcotics. Richards (13) is inclined to the view that the effects of certain neurotoxic insecticides are due to the destruction of the bound lipid sheaths of the nerve cells and their processes. 2. Nerve lesions are of primary importance, according to another school of thought. Death is due to the destruction of certain cells in the central nervous system resulting in functional disturbance of the vital organs.

That nerve lesions possibly may be more an indication of knockdown effect than lethal effect is illustrated by rotenone. The negative report on

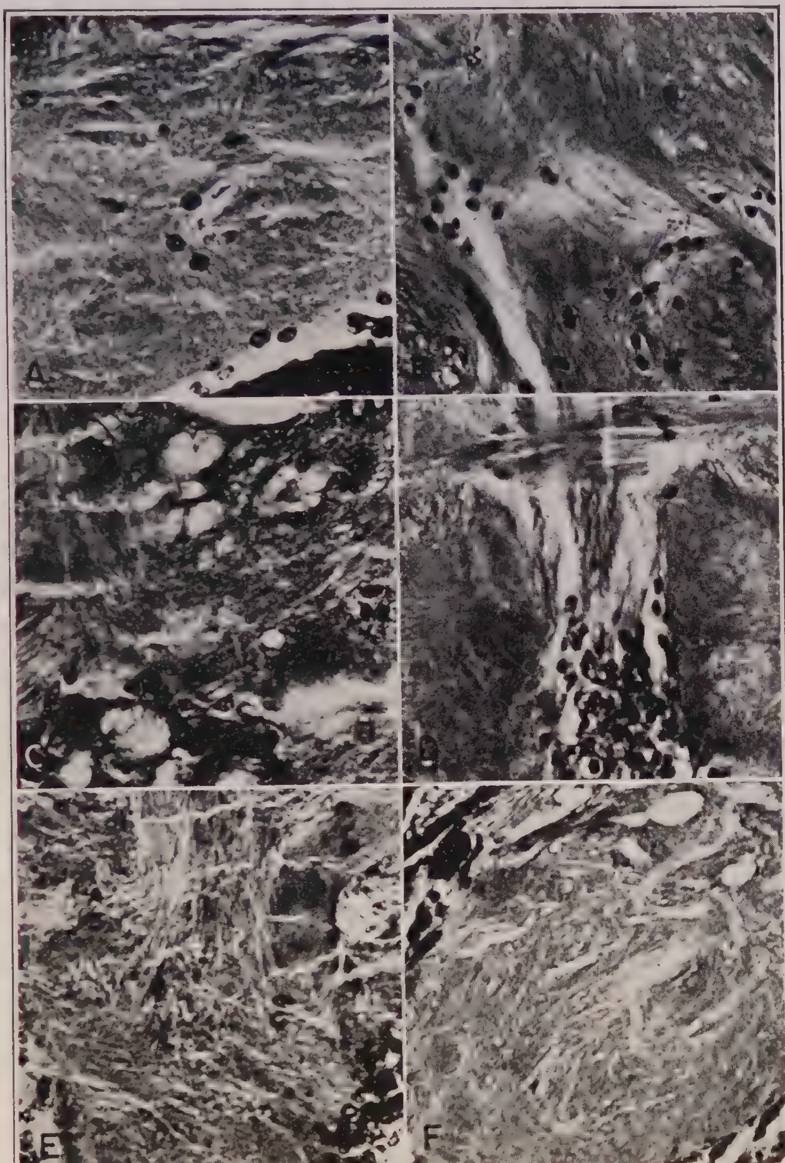

FIGURE 2. Sections through brain of housefly in region of corpus centrale and fiber tracts laterad of it, fixed in formalin and stained with hematoxylin and eosin-y (×500). A to E, 10 min.; F, 1 hr. after spraying. A. Effect of DDT, partial dissolution of fiber tracts and nuclear degeneration. B. Effect of Lethane 384, prominence of nerve fibers and deep-staining nuclei. C. Effect of Thanite, vacuolation around large nerve cells. D. Effect of D.H.S. Activator, similar to B. E. Effect of 0.025% rotenone (10 min.), partial dissolution of fiber tracts, as compared with 1 hr. in F, which in addition shows vacuolation.

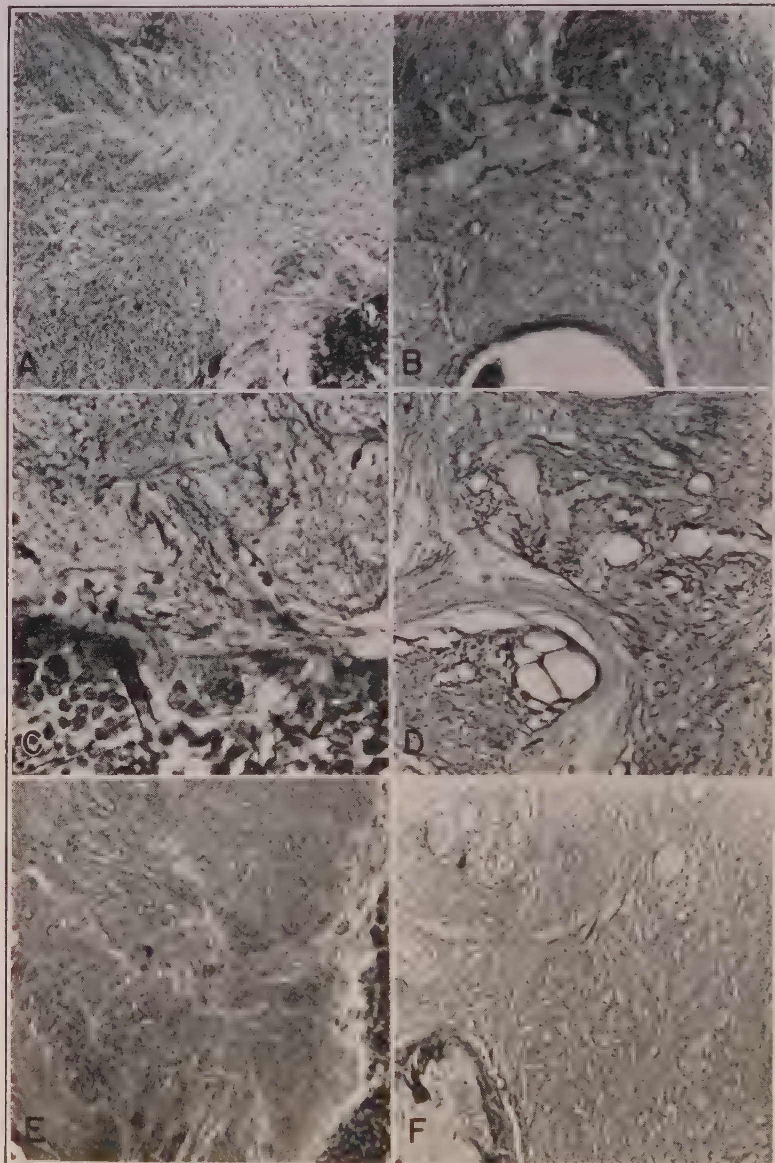

FIGURE 3. Sections through brain of housefly in region of corpus centrale and fiber tracts laterad of it (formalin solution and stained by Bodian's method) (×500). B, C, E, and F, 10 min.; D, 1 hr. after spraying. A. Check. B. Pyrethrum, dissolution of fiber tracts and interrupted tissue showing clear spaces. C. DDT, dissolution of fiber tracts and frayed nerve fibers, 10 min. D. Same treatment as C, but one hr. after spraying, vacuolation around nerve cells and fraying of nerve fibers. E. 0.025% rotenone, dissolution of nerve tracts and vacuolation as compared to 0.00625% in F, with no degeneration.

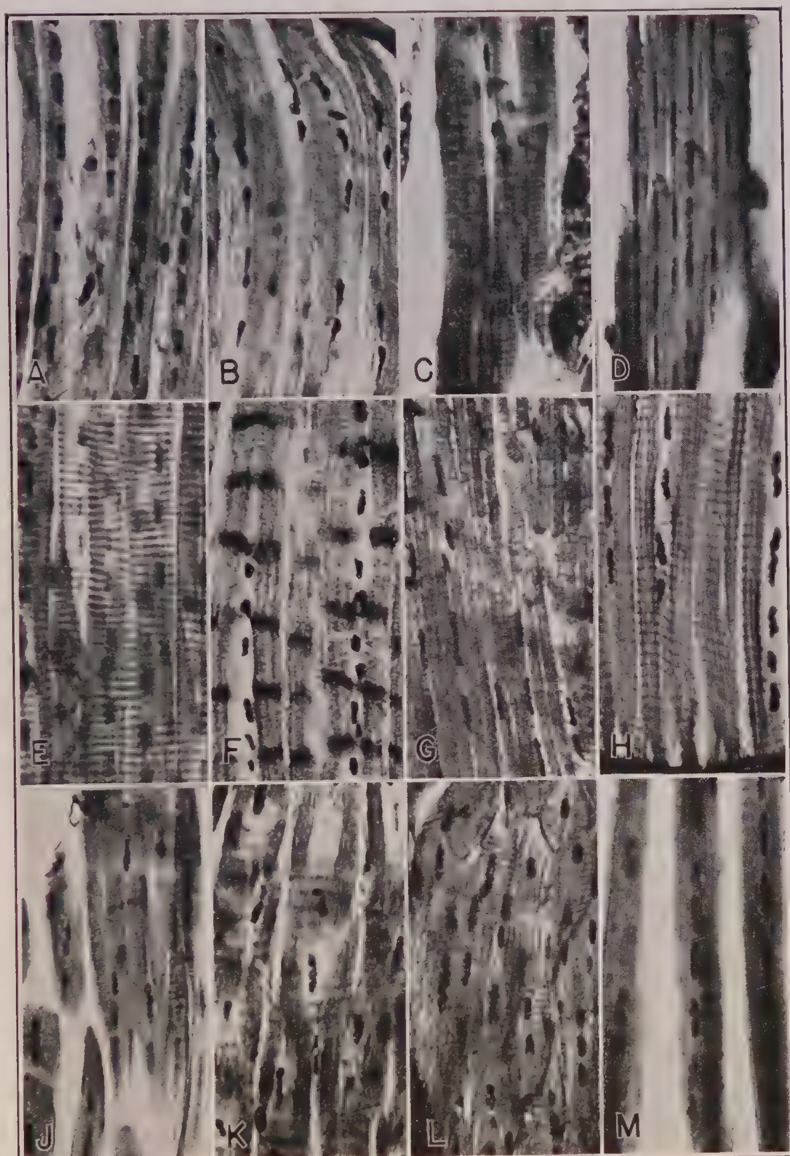

FIGURE 4. Housefly muscle (formalin, hematoxylin+eosin-y) (×500). E and J, 1 hr. after spraying; all others 10 min. A. Check. B. Pyrethrum, nuclei clumped into rod-like dense masses. C. Piperine, slight accentuation of Krause's membrane. D. Piperine+pyrethrum, similar to B. E. Sesame oil, nodes and Krause's membrane greatly accentuated. F. Sesame oil+pyrethrum, clumped nuclei and prominent bands. G. DDT, similar to B. H. D.H.S. Activator, similar to E. J. Thanite, little or no change. K. Lethane 384, destruction of nuclear membranes and muscle strands. L. 0.05% rotenone as compared with 0.025% in M, no degeneration.

rotenone by Klinger (7) as a nerve poison may have been due to the relatively low concentrations applied. The writer's (2) previous experience with rotenone at low concentrations also was negative.

Three organic thiocyano-containing insecticides are now known to produce nerve lesions, namely Lethane 384, Thanite, and γ-thiocyanopropyl phenyl ether (5).

That DDT is a nerve poison is now generally accepted, but that it produces nerve lesions in higher animals is a subject of dispute. Lillie and Smith (8) report vacuolation around large nerve cells in the cord and cerebral motor nuclei in cats, rats, and rabbits. On the other hand, Nelson *et al.* (11) report that no changes can be seen in higher animals that were not present in the controls.

There is evidence from the present investigation and our previous study (3) that activation is due to the destruction of at least two tissue components, the poison having an affinity for one component and the activator for another component and both at lower concentrations than when used alone.

SUMMARY

The effects of eight fly sprays and activators were observed in histological preparations of nerve and muscle tissues of adult houseflies that were sprayed under Peet-Grady test conditions. Flies were selected that would elicit some minor response upon stimulation but were unable to fly, i.e., moribund individuals.

The tissues examined were mainly of the brain, thoracic ganglia, and also from a pair of muscles that run from the occiput to the base of the proboscis on either side of the caudal part of the brain.

The stains used in this histological study were hematoxylin and eosin-y, Bodian's gold chloride and silver albumose method, and toluidine blue.

Pyrethrum and two activators of pyrethrum, namely sesame oil and piperine, were found to produce characteristic effects on the brain of the housefly. The destruction of the fiber tracts and the separation of the tissue by clear spaces are characteristics of pyrethrum poisoning histologically.

With piperine poisoning the nerve fibers are very prominent with almost complete destruction of the cellular components.

Sesame oil when used alone produced effects in the nerve cells at extremely high concentrations, but when used at regularly recommended strengths in combination with pyrethrum produced striking histological effects including vacuolation of the brain tissue and degeneration of the nerve cells.

The effects of Lethane 384 and D.H.S. Activator on brain tissue were similar to that of piperine in that the nerve fibers were very prominent.

Rotenone at lethal concentrations produced no observed histological effect except at concentrations sufficient to cause "knockdown," in which case vacuolation of nerve tissue was pronounced.

DDT caused slight dissolution of the fiber tracts and degeneration of the nuclei both in the brain and in the fused thoracic ganglia, but in spite of its pronounced neurologic symptoms histological changes have been relatively slight.

The muscles of moribund flies following treatment with pyrethrum showed the nuclei clumped into rod-like dense masses due to the clustering of the chromatin.

In the case of flies treated with sesame oil, the muscle nodes and Krause's membrane were greatly accentuated. D.H.S. Activator produced a similar effect on the muscles, while these effects were less pronounced with piperine.

Muscles of moribund flies that had been treated with Lethane 384 showed destruction of the nuclear membrane.

Little or no pathologic change was observed in the muscles of moribund flies that had been treated with rotenone.

Evidence is presented that activation is due to the destruction of at least two tissue components, the poison having an affinity for one component and the activator having an affinity for another component and both at lower concentrations than when used alone.

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AGRONOMIC RELATIONSHIPS OF SODIUM CYANIDE[1]

M. M. McCool

An investigation of some agronomic relationships of sodium cyanide (NaCN) has been completed, namely, soil relationships, fertilizer value, effect on the development of microorganisms, nematodes, and the eradication of undesirable plants. This report deals with the effect of this salt on the pH value of soils, the formation of nitrate nitrogen and ferrous iron in soils, and also on the loss of hydrocyanic acid when it is added to soils.

MATERIALS AND METHODS

In the reaction studies sodium cyanide was applied in solution to 20 grams of soil in Erlenmeyer flasks, a few drops of chloroform added, the flasks shaken several times during a 48-hour period, and the pH value determined by means of a glass electrode.

The rate of nitrate nitrogen formation was determined in the usual manner, namely, by adding to and mixing the cyanide solution with 200 grams of soil, moisture content 20 per cent, and placing the mixture in Erlenmeyer flasks of 500-ml. capacity. The flasks were loosely plugged with cotton and incubated at a temperature of 25° C. At the close of the tests, the contents of each container were mixed, 50-gram samples (dry weight basis) shaken five minutes with 250 ml. of distilled water, filtered, and the nitrates determined colorimetrically.

The ferrous iron released was measured after the method of Ignatieff (2). It should be noted that untreated soil was extracted with aluminum chloride, filtered, and sodium cyanide added to the extract at the rates of 0, 100, 200, and 500 parts per million. The buffer solution and the dye were added and the ferrous iron content determined colorimetrically. The cyanide did not alter the results obtained. The amount of HCN given off was measured by drawing air through a column of a 4 per cent solution of potassium hydroxide, over the soil and again through hydroxide solution. The hydroxide solution was diluted to 0.1 N, 7 ml. of 6 N ammonium hydroxide added to each 100 ml., 0.2 gram of potassium iodide was added, and the solution titrated with 0.1 N silver nitrate (3, p. 238–239). The soil containers were wide-mouthed bottles 5-1/4 inches in diameter and 10 inches in length. Twenty-three hundred grams of the Gloucester loam and 3000 grams of the Leon sand were added to the containers. This resulted in a column of soil about 7 inches in length.

[1] This study was conducted in cooperation with the Electrochemicals Department of E. I. du Pont de Nemours & Co., Inc., of Niagara Falls, New York.

RESULTS OF LABORATORY TESTS

Sodium cyanide is an alkali salt, hence should bring some of the organic matter fraction of the soil into solution when added to it. It was observed that a dark brown deposit of organic matter gradually formed on the surface of the Gloucester loam in greenhouse experiments. Where the B horizon of the Gloucester loam, however, was treated with sodium cyanide a greenish colored deposit resulted. It thus appears that complex reactions may take place when sodium cyanide comes into contact with the soil.

Effect of sodium cyanide on pH. The results (Table I) derived from the tests show that the addition of 50 parts per million of the sodium cyanide

TABLE I

EFFECT OF SODIUM CYANIDE ON THE pH VALUE OF SOILS

Soil treatment sodium cyanide, p.p.m.	Soil type			
	Leon sand	Norfolk sand	Culvers	Lordstown
0	6.10	5.08	4.54	4.95
50	6.40	5.93	—	—
100	6.90	6.20	4.60	5.00
200	7.50	6.93	4.70	5.18
400	—	—	5.00	5.50

to the Leon and Norfolk sands increased the pH values somewhat and 100 parts per million elevated the value of the former to a point near neutrality and of the latter to 6.2. The presence of 200 parts per million resulted in readings of 7.5 and 6.93, respectively. The effects were less striking with the Culvers and Lordstown soils. The addition, for example, of 200 parts of the salt to one million altered the pH values of the soils about 0.2 and 400 parts per million raised the values 0.46 and 0.55, respectively.

Effect of sodium cyanide on formation of nitrate nitrogen. The effect of sodium cyanide on the formation of nitrate nitrogen in soil is demonstrated by Figure 1. The addition of 50 and 100 parts per million, respectively, of cyanide resulted in an increase of the nitrate content of the soil above that in the control cultures, at the close of the 15- and 30-day periods. The presence of 200 parts per million retarded the formation of nitrate nitrogen during the first period but later resulted in an increase in the amount of it. Nitrate formation was retarded during the first period but it was increased during the next or 30-day period in the cultures to which were added 400 parts per million of the sodium cyanide. The increase was less striking than that which was caused by the presence of 200 parts per million of the cyanide.

Formation of ferrous iron by sodium cyanide. According to the results which comprise Table II, the addition of sodium cyanide to Culvers, Langford, Lordstown, Merrimac, Podunk, Sassafras, Leon sand, and

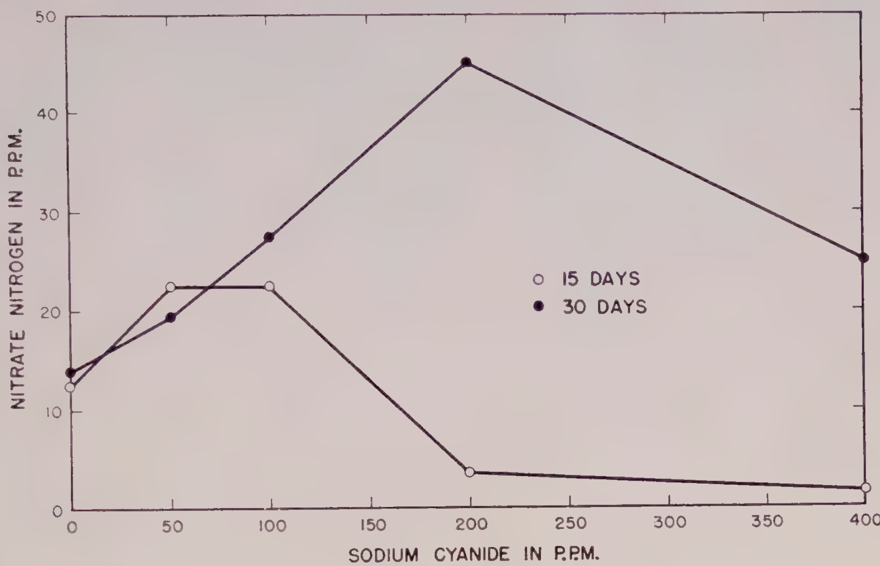

Figure 1. Effect of sodium cyanide on formation of nitrate nitrogen in soil.

Weatherfield soils increased the amount of extractable ferrous iron in them. It should be noted that the soils and cyanide solutions were brought to the desired temperature before they were mixed and held there during the progress of the experiments. The amount extracted varied with the soil type and the temperature under which the tests were conducted.

TABLE II

Effect of Temperature on the Removal of Iron from Soils by Sodium Cyanide. Results Expressed in P.p.m. of Ferrous Iron in Soil. Duration of Test 6 Hours. Ratio of Sodium Cyanide to Soil, 200 Parts per Million

Treatment	NaCN			H_2O		
	Temperature			Temperature		
	5° C.	20° C.	29° C.	5° C.	20° C.	29° C.
	P.p.m. in soil	P.p.m. in soil	P.p.m. in soil	P.p.m. in soil	P.p.m. in soil	P.p.m. in soil
Culvers	2.625	3.150	4.760	0.455	0.665	0.700
Langford	0.805	1.085	1.925	0.210	0.240	0.350
Lordstown	0.525	0.875	1.225	0.350	0.420	0.595
Merrimac	0.700	0.840	2.050	0.250	0.350	0.420
Podunk	0.315	0.420	1.960	0.010	0.020	0.035
Sassafras	0.700	0.805	0.875	0.245	0.280	0.420
Leon sand	0.525	0.700	0.770	0.175	0.350	0.420
Weatherfield	0.175	0.210	0.280	0.035	0.070	0.090

Different amounts of sodium cyanide were added to Merrimac, Dutchess, Langford, Leon sand, Sassafras, and Lordstown soils, at a temperature

of 20° C. After five minutes the amounts of ferrous iron extracted were as given in Table III. According to these data, significant increases in the amount of ferrous iron resulted as the quantities of cyanide were increased. The amounts released, however, were small.

TABLE III

RELEASE OF FERROUS IRON FROM SOILS BY NaCN

Soil treatment NaCN, p.p.m.	P.p.m. ferrous iron in soil					
	Leon sand	Merrimac	Dutchess	Langford	Sassafras	Lordstown
0	1.02	0.52	0.52	0.70	1.08	0.52
100	1.22	0.52	0.87	0.87	1.08	0.52
200	1.40	0.70	1.08	1.08	1.40	0.87
400	1.52	0.85	1.20	1.40	1.62	1.08

Attention should be called to the failure of aluminum chloride to release ferrous iron from the B horizon of Gloucester loam, either with or without the presence of cyanide. It would appear, therefore, that the organic matter fraction of the soil is involved, in part at least, in the release of ferrous iron by extraction with aluminum chloride. Plant growth tests, planned to determine whether chlorosis due to iron deficiency may be prevented by the use of sodium cyanide, are in progress.

Release of hydrocyanic acid by sodium cyanide. The addition of sodium cyanide to the soil results in the release of hydrocyanic acid. Studies on the effect of the water content, pH value of soil, and the placement of the salt in the soil on the loss of this acid have been conducted.

It should be noted that Hitchcock *et al.* (1) experimented extensively with the toxicity of illuminating gas containing hydrocyanic acid in soils toward plants. They report the loss of toxicity to have been rapid when the gassed soil was stored in open containers at temperatures ranging from 20° to 80° C. The loss was much slower in soils which were in sealed receptacles, but even in this case the loss was greatest at the higher temperatures. Leaching with large volumes of water was required to reduce the toxicity. In addition the loss of toxicity appeared to have resulted mainly from the disappearance of hydrocyanic acid.

According to the data in Table IV, the water content of Gloucester loam not only influenced the rate of loss of hydrocyanic acid, but also the quantity of the gas. The first two days of the test 115.5 milligrams of HCN were collected from the soil which contained 10 per cent water, 61.8 mg. from the soil with 20 per cent, 0.5 mg. from the soil with 25 per cent, and none from the soil which carried 28 per cent water. At the close of each of the successive periods, the differences were less striking. The total amount of hydrocyanic acid released was greatest from the soil which contained the smallest amount of water. It should be noted also that small

amounts were released 20 days after the inauguration of the tests. In this table also are given data which bear on the loss of hydrocyanic acid from containers which were kept sealed 10 days, the water content of the soil being 20 per cent. Less than one-half as much acid was collected as was

TABLE IV

Effect of Water Content of Gloucester Loam and Leon Sand on Loss of HCN; 0.5 Gram Sodium Cyanide Added

Length of aspiration period in days	Milligrams of HCN				
	Gloucester loam, water content in per cent				
	10	20	20*	25	28
2	115.5	61.8	34.0	0.5	0.0
2	55.1	57.8	16.7	45.2	1.0
2	21.6	15.7	8.6	28.1	1.6
2	6.5	10.8	3.7	11.3	3.2
2	4.3	3.8	3.2	4.2	6.5
2	1.0	0.5	0.5	1.1	5.4
Total	204.0	150.4	46.7	90.4	17.7
	Leon sand, water content in per cent				
	5	10	15	20	
2	33.5	33.5	33.5	1.1	
2	41.6	41.6	40.0	3.7	
2	24.3	24.3	27.7	4.7	
2	15.1	15.0	13.0	6.5	
2	5.4	5.4	6.5	5.9	
2	4.3	4.3	5.4	5.4	
Total	124.2	124.1	126.1	27.3	

* Salt added, container sealed 10 days and then aspirated.

given off from the soil which was placed in the series immediately after the sodium cyanide was added to it. Where the water content of Leon sand was near the saturation point, as in the case of Gloucester loam, the release of hydrocyanic acid over a 12-day period was relatively small in amount. In order to provide an avenue for the escape of the gas, a hole one inch in diameter was made in the column of soil. Upon aspiration for a period of 48 hours, only 4.8 milligrams of hydrocyanic acid were collected. The amount released from the soil which contained 5, 10, and 15 per cent water, respectively, did not vary significantly.

Sodium cyanide also was added to the surface of Gloucester loam, which was in the air-dried condition, and the rate of release of hydrocyanic acid measured. As the data in Table V show, about as much gas was released over a 6-hour period as was collected during either a 24- or 48-hour period.

The effect of the reaction of Gloucester loam on the liberation of hydrocyanic acid when treated with sodium cyanide was measured. As the data

TABLE V

RATE OF LOSS OF HCN FROM GLOUCESTER LOAM, AIR-DRIED CONDITION; 0.04 GRAM SODIUM CYANIDE ADDED TO 200 GRAMS

Number of tests	Hours aspirated	Milligrams HCN	
		Replicates	
1	6	9.0	9.2
2	24	9.4	10.2
3	48	10.0	10.4

which comprise Table VI show, the acid was released from the soil over a wide range of pH values. Thus, in the use of sodium cyanide for the control of insect pests and plant diseases, the release of hydrocyanic acid under an extensive range of soil conditions should be anticipated.

TABLE VI

EFFECT OF PH OF GLOUCESTER LOAM ON LOSS OF HCN; 0.125 GRAM SODIUM CYANIDE ADDED TO 200 GRAMS. MOISTURE CONTROL OF SOIL 20 PER CENT. PERIOD OF ASPIRATION 2 DAYS

pH	Milligrams HCN
4.25	44.3
5.5	46.4
6.0	46.4
7.0	44.2
7.5	43.6

In order to ascertain whether the soil type affects the amounts of hydrocyanic acid released from soils, 100 grams each of Webster, Podunk, Weatherfield, and Leon sand soils were placed in Erlenmeyer flasks of 500-ml. capacity. The first three were brought to the moisture content of 20 and the Leon sand to 10 per cent, and 0.125 gram of sodium cyanide

TABLE VII

EFFECT OF SOIL TYPES ON RELEASE OF HYDROCYANIC ACID FROM SODIUM CYANIDE; 0.125 GRAM CYANIDE MIXED WITH 100 GRAMS

Soil	Milligrams HCN released in 48 hours
Webster	44.5
Podunk	40.5
Weatherfield	45.9
Leon sand	47.5

added to each. The flasks were sealed immediately with rubber stoppers, the contents shaken, and the amount of hydrocyanic acid given off determined in the usual manner. According to the data which comprise Table VII, the release of hydrocyanic acid from these soils over a 2-day period did not vary significantly.

SUMMARY AND CONCLUSIONS

Sodium cyanide raised the pH values of Norfolk and Leon sands, Culvers, and Lordstown soils.

The addition of 50 and also 100 parts per million of sodium cyanide to Gloucester loam increased the rate of nitrate formation and 200 and 400 parts at first retarded the rate but later increased it.

Increase in the ferrous iron in several soils resulted from the addition of sodium cyanide to them.

Hydrocyanic acid was released when sodium cyanide was added to soils. The rate of loss and also the total quantity given off were greatest in the Gloucester loam with a low water content, and least where the water content was high. The rate and amount of the release from Leon sand did not vary significantly until the water content of the soil approached the saturation point. The loss from Webster, Podunk, and Weatherfield soils and Leon sand did not vary significantly when the water content was similar.

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EFFECT OF SODIUM CYANIDE ON NUMBER OF FUNGI, BACTERIA, AND ACTINOMYCES IN SOIL AND ITS VALUE IN THE CONTROL OF DAMPING OFF OF SEEDLINGS, NEMATODES, AND CABBAGE ROOT WORM[1]

M. M. McCool

Tests have been made on the effect of sodium cyanide on the number of fungi, bacteria, and *Actinomyces* in the soil and on the control of organisms which cause the damping off of seedlings and the control of nematodes and cabbage root worm. The results derived from these tests show that the total number of microorganisms in soil may be reduced temporarily, damping off of seedlings reduced, and nematodes controlled by this salt.

LITERATURE REVIEW

Tam and Clark (7) added calcium cyanide in the form of the commercial product, Cyanogas, to soil at the rate of 80 parts of nitrogen per million. They present a review of literature and report complete elimination of nitrifying organisms in the soil by this material. The cyanide had relatively little effect on fungi and *Actinomyces* but the total number of bacteria was increased by it.

Campbell (2) employed granular calcium cyanide to control wireworms in the soil. After the use of bean or wheat in rows to act as bait for the worms, six pounds of the cyanide were applied per 1000 feet about one-half inch below the bait. He maintains that any crop may be planted about five days later. Plants which are in hills and which may be attacked by the wireworms may be protected by applying about one teaspoonful of the cyanide in a hole which is then filled with soil. The soil is compacted slightly.

Sodium cyanide (NaCN) has been employed successfully for the eradication of certain plant diseases by Milbrath (6), and nematodes by Tyler (8), Watson (9, 10), and Duruz (3).

McCallan and Weedon (4) in their studies on the toxicity of SO_2, Cl_2, HCN, NH_3, and H_2S found HCN to be the least toxic toward eight plant pathogens and two animal pathogens and also noted a reciprocal between time and concentration.

Barton (1) studied the effect of hydrogen cyanide on the time required for germination and also on the percentage of germination of radish and rye seeds. The seeds were placed on moist filter paper in Petri dishes. She reports a delay in germination of the soaked seeds under some conditions.

[1] This study was conducted in cooperation with the Electrochemicals Department of E. I. du Pont de Nemours & Co., Inc., of Niagara Falls, New York.

METHODS OF PROCEDURE

The plate culture method was employed in determining the effect of sodium cyanide on the number of fungi, bacteria, and *Actinomyces* in the soil. One hundred grams of soil, moisture content 20 per cent, were placed in Erlenmeyer flasks of 500-ml. capacity, treated with the cyanide, shaken and plugged with cotton. The cultures were held at the temperature of 28° C. until samples were taken for planting. Pure cultures of fungi which cause the damping off of seedlings in the soil were employed, 50 grams of soil were placed in Erlenmeyer flasks, treated with 10 ml. nutrient solution, the flasks plugged with cotton, and sterilized in an autoclave daily for three days. Different amounts of sodium cyanide were added to the containers and the contents were then inoculated with the fungi. This was accomplished by breaking up the fungal growth with sterile sharp sand and adding 1 ml. to the soil. Each treatment was replicated four times. Where the effect of hydrocyanic acid was measured the fungi *Cunninghamella blakesleeana* Lindner and *Alternaria* sp. (from tomato) were employed. Tubes of agar were inoculated with the fungi, and were placed in Mason jars of one-quart capacity to each of which 10 grams of Gloucester soil in the air-dry condition had been added. The jars were sealed immediately after the addition of sodium cyanide with rubber gaskets over which was placed molding clay. The seals were broken in one set of containers after 48 hours, in order to permit the escape of hydrocyanic acid.

In the first series of tests in which the effect of sodium cyanide on seed germination in soil infested with organisms which cause the damping off of seedlings was measured, the soil was screened, placed in containers, sodium cyanide added, and the mass shaken in order to mix the materials. After 48 hours the treated and untreated soils were placed in potting frames. The room in which the tests were conducted was maintained at the temperature of 16° C. The infested soil used in these tests was obtained through the courtesy of Dr. E. E. Clayton, Bureau of Plant Industry, U. S. Dept. of Agric., Washington, D. C.

Additional tests were made in which the cyanide was mixed with a small amount of quartz sand in order to facilitate its application. The mixture was placed about one-sixteenth of an inch below the seed in the row. The seed employed in these studies were tobacco (*Nicotiana tabacum* L.), cabbage (*Brassica oleracea* L.), and pea (*Pisum sativum* L.).

The soil which was heavily infested with nematodes and which came from Maryland was treated with granular sodium cyanide. The salt was placed one inch from the bottom of the containers, and a seven-inch layer of soil placed above it. The containers, tin cans of five-gallon capacity, were sealed and so remained 72 hours. At the close of this period the contents were shaken, removed, mixed and spread in a thin layer and so remained until the moisture content had reached 10 per cent. Then it was

placed in glazed jars of one-half gallon capacity. Tomato plants were set in the cultures.

The tests on the control of cabbage root worms were carried on in the field. The sodium cyanide was mixed with the seed bed to a depth of six inches. Cabbage plants were set in the soil one week after the addition of the cyanide.

EXPERIMENTAL RESULTS

Soil from a vegetable garden was treated with different amounts of sodium cyanide and counts with respect to the number of fungi, bacteria,

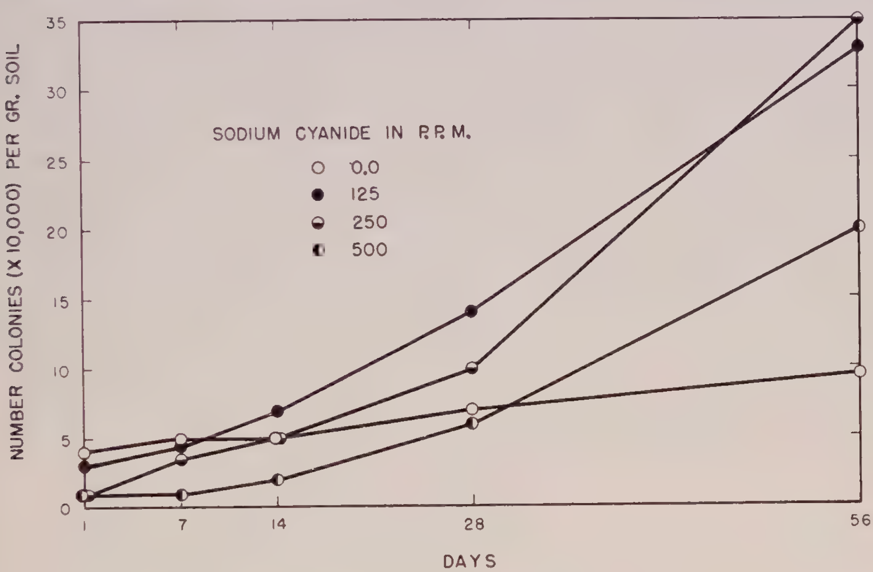

Figure 1. Effect of sodium cyanide on the number of fungi in soil.

and *Actinomyces* were made after different intervals of time. According to the results represented by Figure 1, the addition of 250 and 500 parts of cyanide at first reduced significantly the number of fungi, and later each increment resulted in greater numbers. Later the numbers were greater in the treated soil than they were in the controls.

As the amount of cyanide added to the soil was increased the time required for such to occur lengthened. It may be cited, for example, that the number of fungi in the cultures which received 125 parts per million of the cyanide showed an increase after 14 days and after 28 and 56 days, where 250 and 500 parts per million respectively were added to the soil.

Counts of bacteria and *Actinomyces* were made in untreated soil and that treated with sodium cyanide. According to the data which comprise Table I, the addition of 25 parts per million of sodium cyanide to garden

soil increased the total number of these organisms, whereas 50 and 100 reduced them, and 200 and 400 parts per million prevented their development. It was brought out (5) that the activities of nitrifying bacteria were prevented temporarily by the addition of this salt to the soil.

TABLE I

EFFECT OF SODIUM CYANIDE ON THE NUMBER OF BACTERIA AND ACTINOMYCES IN GARDEN SOIL. RESULTS GIVEN IN MILLIONS OF COLONIES IN 1 GRAM OF SOIL, AVERAGE OF FOUR REPLICATES 2 DAYS AFTER ADDITION OF CYANIDE TO SOIL

Treatment, p.p.m. cyanide	Replicates, No. of colonies				Av. No. of colonies
0	7	6	4	7	6.0
25	11	14	12	13	12.5
50	2	5	3	4	3.5
100	1	2	1	3	1.7
200	0	0	0	0	0
400	0	0	0	0	0

Tubes of agar were inoculated with fungi, *Alternaria* and *Cunninghamella*, and placed in glass Mason fruit jars to which 10 grams of soil had been added. Different amounts of sodium cyanide in solution were added to the soil and the containers sealed at once. The seals were broken after 48 hours in one set. Some of the growth records comprise Table II. It is to be observed that the addition of 0.004 and 0.006 gram of sodium cyanide liberated sufficient hydrocyanic acid to prevent the development of the fungi in the tubes of agar. Although they are not given in the table, the daily records with respect to the first six reveal that the development of

TABLE II

EFFECT OF HYDROCYANIC ACID ON GROWTH OF FUNGI ON STERILE AGAR IN SEALED CONTAINERS

Treatment, NaCN in grams per container	Development			
	Sealed containers		Transferred to sterile agar	
	Alternaria	*Cunninghamella*	*Alternaria*	*Cunninghamella*
0	+	+	+	+
0.001	+	+	−	−
0.002	+	+	+	+
0.004	0	0	+	+
0.006	0	0	+	0

Alternaria was discernible one day after the tests were started in the control containers, two days later in those which contained 0.001 gram, and four days later in those to which 0.002 gram was added. The rate of the subsequent growth of the fungi was also delayed. The appearance of the *Cunninghamella*, a very rapidly growing fungus, was noted 24 hours after inoculation in the untreated tubes of agar and two and five days after, respectively, in those which contained 0.001 and 0.002 gram of the cyanide.

The results derived from the second lot of tubes where the seal was broken did not differ significantly from those just mentioned. An additional series of tests was conducted. Here transfers from the tubes in the sealed containers to sterile tubes of agar were made. According to the results presented in Table II, the fungi developed in each transfer with the exception of *Cunninghamella* which came from the containers to which 0.006 gram of sodium cyanide had been added. The development of the fungi was obvious 24 hours after transfer from the controls and those containers treated with 0.002 gram of the cyanide. After 72 hours, the fungi had developed in those which were taken from the containers treated with 0.004 gram of

TABLE III

EFFECT OF SODIUM CYANIDE ON GROWTH OF FUNGI

Treatment, p.p.m. cyanide	Fungus* 1	2	3	4	5	6
	Containers not sealed					
0	+	+	+	+	+	+
200	+	+	+	+	+	+
400	+	+	+	+	+	+
800	0	+	0	0	+	0
1000	0	0	0	0	0	0
1200	0	0	0	0	0	0
	Containers sealed 48 hours					
0	+	+	+	+	+	+
50	+	+	+	+	+	+
100	+	+	+	+	+	+
150	0	0	0	0	0	0
200	0	0	0	0	0	0
400	0	0	0	0	0	0
500	0	0	0	0	0	0

* 1. *Phytophthora* (tomato); 2. *Pythium*; 3. *Rhizoctonia*; 4. *Phytophthora* (Swiss chard); 5. *Alternaria*; 6. *Verticillium*.

the cyanide. After 96 hours, growth of *Alternaria* had taken place in those removed from the receptacles to which 0.006 gram of sodium cyanide had been applied, but four days later, or after 192 hours, when the tests were terminated there was no evidence of growth of *Cunninghamella*.

Sterilized soil to which nutrient medium had been added was inoculated with each of several fungi (*Phytophthora* sp. [from tomato], *Pythium* sp. [from Swiss chard], *Rhizoctonia* sp. [from Swiss chard], *Phytophthora* sp. [from Swiss chard], *Alternaria* sp. and *Verticillium alboatrum* Reinke & Berth.) which cause the damping off of seedlings. The flasks were treated with different amounts of sodium cyanide. In one set the containers were plugged with cotton in the usual manner and in another the flasks were sealed immediately after treatment and kept sealed for 48 hours. According to the results given in Table III, between 800 and 1000 parts per million

of sodium cyanide were required to prevent the appearance of the fungi in the first set of tests and between 100 and 150 parts per million in the second series or where the containers were sealed for 48 hours.

TABLE IV

EFFECT OF SODIUM CYANIDE ON GROWTH OF CULTURES TRANSFERRED FROM SOIL TO STERILE AGAR AFTER DIFFERENT TIME PERIODS IN HOURS

Treatment, p.p.m. cyanide	*Cunninghamella*				*Alternaria*				*Macrosporium*			
	6	24	48	96	6	24	48	96	6	24	48	96
0	+	+	+	+	+	+	+	+	+	+	+	+
50	+	+	+	+	+	+	+	+	+	+	+	+
100	+	+	+	+	+	+	+	+	+	+	+	+
150	+	+	+	0	+	+	+	0	+	+	+	0
200	+	+	+	0	+	+	+	0	+	+	+	0

An additional series of tests was run in order to ascertain whether the exposure of fungi to sodium cyanide in soil merely inhibited or killed them. Three organisms, namely *Cunninghamella*, *Alternaria* (from tomato), and *Macrosporium* (isolated from cabbage) were employed as the indicators. Cultures were transferred from the treated soil to tubes of sterilized agar, after 6, 24, 48, and 96 hours. The data derived from these tests comprise Table IV. The addition of cyanide at the rate of 150 and 200 parts to one million of soil (exposure 96 hours) prevented the growth of the fungi. Shorter periods of exposure to the cyanide in the soil in the sealed containers did not prevent their development.

TABLE V

EFFECT OF SODIUM CYANIDE ON SEED GERMINATION IN SOIL INFESTED WITH DAMPING OFF ORGANISMS, 50 CABBAGE AND 20 PEA SEED PLANTED IN EACH REPLICATE

Cultural treatment	Cabbage					Pea				
	Germination per cent					Germination per cent				
	Replicates				Av.	Replicates				Av.
No treatment	14	16	10	12	13	0	0	0	0	0
Steamed soil	85	90	85	80	85	85	80	75	80	80
Seed dusted with 2,3-dichloro α-naphthaquinone						75	80	75	80	77.5
Seed dusted with Semesan	86	84	82	80	83					
100 p.p.m. cyanide	16	18	14	12	15	0	0	0	0	0
200 " "	74	80	76	78	77	80	75	70	70	73.7
300 " "	70	82	80	78	77.5	80	75	80	70	76.2

The effect of sodium cyanide on the germination of seed in soil from Maryland, known to be heavily infested with organisms which cause the damping off of seedlings, was studied. The results derived from these tests comprise Table V. Here the cyanide was mixed with the soil in closed con-

tainers. After 48 hours the soil was placed in the flats and the seed planted. Increase in the number of cabbage and pea seed germinated resulted in the soil which received 200 and also 300 parts per million of the sodium cyanide. The cyanide compared favorably with Semesan and 2,3-dichloro α-naphthaquinone in the reduction of the activities of the organism which caused damping off of the seedlings.

In an additional series of tests, the sodium cyanide was placed in rows below the seed. The data derived from these tests comprise Table VI. Each increment of cyanide resulted in an increase in the number of seed germinated. The presence of 0.09 gram increased the percentage of cabbage from 15 in the untreated soil to 41, 0.12 gram to 56.2, and 0.15 gram to

TABLE VI

Effect of Sodium Cyanide on Seed Germination in Soil Infested with Damping Off Organisms, 40 Cabbage and 100 Tobacco Seed Planted in Each Replicate

NaCN gram per linear foot	Germination per cent						
	Cabbage						Av.
0	17.5	12.5	15	22.5	10	12.5	15
0.06	22.5	15	12.5	12.5	32.5	17.5	18.7
0.09	37.5	31	27.5	30	70	50	41
0.12	60	62.5	47.5	42.5	60	65	56.2
0.15	57.5	50	72.5	52.5	57.5	67.5	59.5
	Tobacco						
0	8	12	8	4	7	6	7.5
0.075	30	19	22	38	26	30	27.5
0.10	47	60	28	18	15	20	31.3
0.125	55	40	30	27	48	51	41.8

59.5 per cent. Seven and one-half per cent of the tobacco seed germinated in the untreated soil and the addition of 0.075, 0.10, and 0.125 gram of sodium resulted in averages of 27.5, 31.3, and 41.8, respectively.

Sodium cyanide proved to be effective also in the control of nematodes, which greatly retarded the development of tomato plants in a soil from Maryland. The roots of the plants which were grown in the control soil and that to which had been added 50 parts per million of cyanide, respectively, were heavily infested with nematodes while those in the soil which received 75 parts per million showed much less injury, and there was no visible evidence that the nematodes had attacked the plants which were placed in the soil to which 100 parts per million and greater had been made. The results are given in Table VII and illustrated by Figure 2.

Sodium cyanide applied to soil at the rate of 400 pounds per acre on the surface area basis, and mixed with the soil to a depth of six inches, failed to prevent the attack of roots of cabbage plants by root worm. Sixteen plants were set in the untreated soil and the same number in the soil

with which the sodium cyanide was mixed. Three plants from each area were not attacked by the cabbage root worm.

TABLE VII

EFFECT OF SODIUM CYANIDE ON NEMATODES. PLANT INDICATOR, TOMATO. DURATION OF TEST, 47 DAYS

NaCN, p.p.m.	Number of nodules on plants		
	1	2	3
0	222	78	140
50	135	153	94
75	51	36	74
100	0	0	0
200	2	0	0
400	0	0	0
800	0	0	0
1600	0	0	0

FIGURE 2. Control of nematodes by sodium cyanide. (Left) Roots of tomato plants grown in soil treated with 100 parts per million of sodium cyanide. (Right) Roots grown in soil treated with 50 parts per million of sodium cyanide.

SUMMARY AND CONCLUSIONS

The number of fungi in soil taken from a vegetable garden was at first reduced by the addition of 250 and 500 parts per million of sodium cyanide to it. After 14 days increase resulted from the use of 125, 28 days from the

addition of 250, and 56 days from the presence of 500 parts per million of the cyanide.

The total number of bacteria and *Actinomyces* in the soil was reduced two days after treatment by the addition of 50 and 100 parts per million and growth prevented by the addition of 200 and 400 parts per million of sodium cyanide to it.

The addition of 0.004 gram or more of sodium cyanide in solution to dry soil liberated sufficient hydrocyanic acid in one-quart Mason jars to prevent the growth of *Alternaria* sp. and *Cunninghamella blakesleeana* fungi in tubes of agar. When transfers were made, however, to sterilized agar, growth resulted in each case within 8 days with the exception of *Cunninghamella* taken from the cultures to which 0.006 gram of cyanide was added.

Six fungi which cause the damping off of seedlings were prevented from developing in soil cultures to which 800 or more parts per million of sodium cyanide were added where the containers were not sealed. When the containers were sealed 48 hours 200 or more parts per million prevented their development.

Cultures of *Cunninghamella*, *Alternaria*, and *Macrosporium* were taken from soil treated with different amounts of sodium cyanide. Growth of each organism took place after they had been in the soil, treated with 150 and 200 parts per million of the cyanide, 6, 24, and 48 hours. Where they were exposed to the treatment 96 hours, however, growth was not discernible six days after the cultures were transferred to sterilized agar.

The damping off of cabbage and pea seedlings was partially controlled by the addition of 200 and 300 parts per million of sodium cyanide to the soil where the containers remained sealed 48 hours after the salt was mixed with the soil.

The placement below the seed of 0.06, 0.09, 0.12, and 0.15 gram of sodium cyanide per linear foot in the order given, partially controlled the damping off of cabbage and 0.075, 0.10, and 0.125 gram that of tobacco seedlings.

Sodium cyanide proved to be effective in the control of nematodes which attacked the roots of tomato plants, where it was applied seven inches below the surface of the soil and where the receptacles remained sealed 72 hours before the soil was removed and the plants set in it.

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USE OF SODIUM CYANIDE FOR THE ERADICATION OF UNDESIRABLE PLANTS[1]

M. M. McCool

Owing to the general interest in the use of chemicals to kill undesirable plants we have conducted experiments on the use of sodium cyanide for eradication of several plants. In some instances it was compared in efficiency with ammonium sulfamate. The results derived from these investigations comprise this report.

METHODS OF PROCEDURE

Solutions of sodium cyanide varying in concentration were utilized in these tests. The plants included in the investigations were dandelion (*Taraxacum officinale* Weber), plantain (*Plantago major* L.), crab grass (*Digitaria sanguinalis* [L.] Scop.), quack grass (*Agropyron repens* [L.] Beauv.), foxtail (*Alopecurus pratensis* L.), European bindweed (*Convolvulus arvensis* L.), hedge bindweed (*Convolvulus sepium* L.), honeysuckle (*Lonicera dioica* L.), privet (*Ligustrum vulgare* L.), gardenia (*Gardenia jasminoides* Ellis), and poison ivy (*Rhus toxicodendron* L.).

Unless otherwise stated, a sprinkling can with fine rose attachment was employed in applying the salt solutions. One liter was added per square meter, as was done by Fromm and Vidal (1) in their experiments on the control of Bermuda grass and crowfoot grass. The grasses with the exception of quack grass were clipped with a lawn mower before the salt solutions were applied. The quack grass was cut by means of a scythe.

EXPERIMENTAL RESULTS

The results derived from the application of the sodium cyanide and ammonium sulfamate to plants are assembled in Table I. The sodium cyanide was applied in the granular form to the crown, solution sprinkled on the surface of the plants, poured on the surface of the soil and into holes adjacent to plantain and dandelion plants. Fifty milliliters of the solutions were poured into the holes which were three-fourths of an inch in diameter and four inches in depth. The effects of the cyanide were visible about 48 hours after its application. According to the data, each of the methods of application was successful in the eradication of these plants. Solutions which carried 2 or more per cent of the sodium cyanide and 0.5, 1, and 2-gram applications of the solid killed the plants. The procedures which were the most satisfactory were the application of the solid to the crowns and the solutions in the holes since by their use a minimum amount of grass adjacent to the plants was killed.

[1] This study was conducted in cooperation with the Electrochemicals Department of E. I. du Pont de Nemours & Co., Inc., of Niagara Falls, New York.

TABLE I

ERADICATION OF PLANTS BY SODIUM CYANIDE AND AMMONIUM SULFAMATE

Plant	Applied to	Relative degree of injury								
		Concentration of NaCN per cent								
		0.5	1	2	3	4	5	6	8	9
Plantain	Solution on surface of soil		No injury	Dead	Dead	Dead	Dead	—	—	—
	Holes adjacent to plant		"	"	"	"	"	—	—	—
Dandelion	Solution on surface of soil		"	"	"	"	"	—	—	—
	Holes adjacent to plant		"	"	"	"	"	—	—	—
Crab grass	Sprinkled on plants		Injured	"	"	"	—	—	Dead	—
Foxtail	"		"	"	"	"	—	—	"	—
Quack grass	"		No injury	Injured	"	"	—	—	—	—
Hedge bindweed	Sprinkled on plants		Slight injury	"	"	"	—	—	—	—
	Holes adjacent to plant		"	"	"	"	—	—	—	—
European bindweed	Sprinkled on plants		"	"	"	"	—	—	—	—
	Holes adjacent to plant		"	"	"	"	—	—	—	—
Poison ivy	Sprinkled on leaves		No injury	Dead	"	"	—	—	Dead	—
	Roots in soil		"	No injury	"	—	Dead	—	—	Dead
Honeysuckle	Sprinkled on leaves		"	Injured	—	Dead	—	—	Dead	—
Privet	Roots in soil	No injury	No injury	Dead	—	—	—	—	—	—
Gardenia	"	Injury	Dead	Dead	—	—	—	—	—	—

Plant	Applied to	Quantity NaCN in grams					
		0.25	0.5	1	2	4	8
Plantain	Solid on crown	Injured	Dead	Dead	Dead	—	—
Dandelion	"	"	"	"	"	—	—

TABLE I (*Continued*)

		Concentration of ammonium sulfamate in per cent				
		1	2	3	4	8
Quack grass	Plants	No injury	Injured	Dead	Dead	—
Crab grass	"	Injured	Dead	"	"	—
Foxtail	"	"	"	"	"	—
Poison ivy	"	No injury	"	"	"	Dead

Gardenia plants growing in porous pots 7 inches in diameter and privet plants derived from cuttings which had been taken one year previously were employed to measure the effect of sodium cyanide on woody plants when applied to the root systems. Two hundred milliliters were applied to the gardenia plants and two liters to the privet plants. As the data in Table I show, gardenia plants were killed by the addition of solutions of 1 per cent and greater. In addition, the terminal clusters of leaves together with the stem to which they were attached were inserted into Erlenmeyer flasks of 150-ml. capacity, which contained a solution of sodium cyanide (0.5 g. of NaCN per 100 ml.) and held there over a period of one week. The leaves and portions of the stem which were in contact with the solution became colorless and the remainder of the leaves attached to the stem in question and those of the branches which it carried were killed. Thus the indications are that the injurious material moved not only downward but laterally and upward in the branch in question.

The privet plants were treated with the sodium cyanide by placing it in holes in the soil. The holes were four inches in depth and six inches in diameter. The hole for each was dug on one side and adjacent to the plant. The results in Table I show the plants to have been killed by a 4 per cent solution.

Crab and foxtail grasses were killed by concentrations of 2 per cent or greater of sodium cyanide and also by ammonium sulfamate of the same concentrations which were applied on August 7. Grasses and weeds appeared about September 25 in the plots to which the cyanide had been applied, but they did not appear before cold weather set in where the sulfamate was added. It should be noted also that the barren soil of the plots which had been treated with 4 and 8 per cent solutions, respectively, of these salts was broken up to a depth of one and one-half inches 15 days after the salts were applied and perennial rye grass seeded. An excellent stand of rye grass resulted on each of the plots.

It appears that the practice of clearing the immediate surface soil of weeds and grasses with chemicals could be followed in establishing a pure stand of a given grass or mixture of grasses.

The above-ground portions of quack grass were killed by the addition of concentrations of 3 per cent and greater of sodium cyanide and also by ammonium sulfamate of the same concentration. The salts were applied on June 28. After about two weeks, quack grass appeared in the plots which had been treated with the cyanide, but the surface of the soil to which the sulfamate was applied remained free of plant growth throughout the remainder of the season.

In another set of experiments 2, 4, and 6 per cent solutions of sodium cyanide were added on July 5 to plots containing quack grass. On August 1 and September 20 the treatments were repeated. The stand of grass was reduced greatly by the first and second applications and there was very little re-growth after the third application. Thus cyanide may be used to eradicate quack grass but repeated applications are necessary. The ammonium sulfamate is more effective than the cyanide but the cost of the materials becomes of importance in the selection of the salt to be used for this purpose.

Tests on the eradication of hedge bindweed and European bindweed from vegetable gardens and also the latter from a rock garden adjacent to a porch of a dwelling were conducted. The vegetable gardens were heavily infested with these weeds and the European bindweed had killed rose plants which were attached to the porch. The leaves of the weeds were drenched with the sodium cyanide on June 27 and the roots were treated during the month of July. According to the results recorded in Table I these weeds were killed by drenching the leaves with cyanide solutions and also by pouring the solutions into holes adjacent to the plants. Solutions of 3 per cent or greater were effective.

Honeysuckle plants were sprinkled with sodium cyanide on July 29. According to the data in Table I, 1 and 2 per cent solutions of this salt did not kill the above-ground portions of these plants, although some injury by the latter was recorded. Four and 8 per cent solutions killed them. No growth appeared in the latter during the remainder of the growing season.

The last plant to be considered is poison ivy. The solutions of sodium cyanide were applied to the leaves and also to the roots on August 15. As the data in Table I reveal, the leaves of the plants (which were growing along a roadside) were killed by solutions which contained two or more per cent of the salt. Additional growth did not take place on these plants during the remainder of the growing season.

It was found that the addition of one liter of either 3, 6, or 9 per cent solutions of sodium cyanide to the roots of plants which were growing on fence posts and trees killed their leaves. About one week was required for the effect of the treatment to be visible in the veins of the leaves. Where the plants had reached the trees by advancing several feet on the ground

the treatment was not successful. Under such conditions it would appear to be advisable to cut the vine at the base of the tree and sprinkle the solution on the leaves as they appear.

SUMMARY AND CONCLUSIONS

Sodium cyanide has been used successfully in the eradication of dandelion and plantain. It was successful whether applied in the solid form to the crowns of the plants, in solution sprinkled on the leaves, poured on the soil around the plants or into holes adjacent to them.

One application of sodium cyanide killed crab grass and foxtail grass, but single applications did not prevent the reappearance of quack grass. Three applications of solutions containing 3 or more per cent of the salt each at the rate of one liter per square meter were required to prevent the reappearing of plants from July until the close of the growing season. One application of ammonium sulfamate in solution containing 3 per cent or more killed quack grass.

Hedge bindweed and European bindweed were killed either by drenching the above-ground portions with solutions of sodium cyanide of 4 per cent or stronger or by pouring solutions into holes adjacent to the plants.

Honeysuckle vines were killed by the use of one liter of 3 per cent solution on each square meter.

Poison ivy plants which were growing along the roadside were eradicated by sprinkling with solutions of 2 or more per cent of sodium cyanide and by applying 3 per cent or greater solutions to the roots of plants, the vines of which had climbed trees and fence posts.

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FERTILIZER VALUE OF SODIUM CYANIDE[1]

M. M. McCool

One phase of the studies on the agronomic relationships of sodium cyanide (1) consisted of an investigation of its fertilizer value. The plant growth tests were conducted in a greenhouse and also in the field. The results show this chemical to delay seed germination somewhat, to leach less readily from soil than sodium nitrate, and to increase crop growth when added to soils deficient in nitrogen. It was less effective in the latter respect than was sodium nitrate.

MATERIALS AND METHODS

Potting frames 3 inches in depth, 13 inches in width, and 18 inches in length were used as soil containers in the seed germination tests. The sodium cyanide was added in solution to and mixed with the soils, the water content of each being 60 per cent of their water-retaining capacity. One hundred seeds were planted in rows in the various containers. Glazed jars of two-gallon capacity were employed as the containers in the growth tests conducted in the greenhouse. Fifty-six hundred grams of Gloucester loam were added to each container. The cyanide solution was mixed with the soil before it was placed in the containers. A basic treatment consisting of 6 grams of superphosphate (16 per cent P_2O_5), 0.5 gram of potassium chloride, and sufficient precipitated calcium carbonate to bring the pH value of the soil to 6.6 was afforded each culture.

Galvanized rims 2 feet in diameter and 8 inches in depth were employed as the containers for the field studies. The nitrogen carriers were mixed with sand and this was incorporated in the upper 6 inches of soil. A basic treatment consisting of a sufficient amount of calcium carbonate to bring the pH value of the soil to 6.4, superphosphate at the rate of 400 pounds per acre, and potassium sulphate at the rate of 200 pounds per acre was given each container. After the fertilizers were applied, four uniform tobacco plants were set in each. The plants were harvested when in the early blossom stage. The plants employed as growth indicators in the greenhouse test were corn (*Zea mays* L. var. Frank Bros. hybrid G-104), lettuce (*Lactuca sativa* L. var. Imperial 44), tobacco (*Nicotiana tabacum* L. var. Turkish), and white mustard (*Brassica alba* Rabenh.). The duration of the growth period in the greenhouse studies of corn was 52, tobacco 52, lettuce 62, and mustard 39 days.

[1] This study was conducted in cooperation with the Electrochemicals Department of E. I. du Pont de Nemours & Co., Inc., of Niagara Falls, New York.

RESULTS

SEED GERMINATION TESTS

The results derived from the tests on the effect of sodium cyanide on the germination of seeds in soils comprise Table I. The addition of 50 parts of sodium cyanide to one million of Leon sand delayed the germination of radish seed about one day, 100 parts per million about two, and 200 parts per million four days. Similar trends were observed with respect to the Gloucester soil although larger amounts were required to delay the germination of the radish seed. The appearance of corn seedlings in Leon sand was delayed to a slight extent by 100 and 200 parts per million and by 200 and 400 in the Gloucester soil; the germination of bean seeds in these soils was delayed by each amount of the sodium cyanide added. The effects were not obvious four days after the seed were planted.

TABLE I

EFFECT OF SODIUM CYANIDE ON PER CENT GERMINATION OF SEED

Soil type	Treatment NaCN added, p.p.m.	Radish						Corn			Bean				
		April						April			April				
		24	25	26	27	28	29	26	27	29	26	27	28	29	30
Leon sand	0	*84*	93	93	93	93	93	96	98	98	94	94	96	98	
	50	23	*86*	92	92	92	92	99	99	99	22	*63*	94	96	
	100	0	40	*82*	89	89	91	*88*	94	97	18	34	*89*	96	
	200	0	3	38	66	*76*	90	*89*	92	96	0	7	*71*	97	
Gloucester soil	0	*73*	86	86	86	86		93	95		*94*	96	96	97	98
	100	2	*69*	89	89	89		98	98		22	*63*	94	94	96
	200	0	*72*	83	83	89		91	99		18	34	*89*	93	96
	400	0	16	48	*76*	83		*79*	97		0	7	71	*90*	97

Note: Italicized figures show delay in seed germination.

FERTILIZER VALUE

The fertilizer value of sodium cyanide in comparison with nitrate of soda and also ammonium sulphate was measured by plant growth tests in the greenhouse and in the field.

Greenhouse tests. The total yields derived from tobacco and corn plants grown in the greenhouse where no nitrogen, 0.16, 0.32, and 0.48 gram of nitrogen in the form of nitrate of soda and sodium cyanide respectively were added, are given in Table II. According to these results each amount of sodium cyanide increased the yield of tobacco and corn plants. It should be noted, however, that the sodium nitrate was superior to the sodium cyanide irrespective of the amount of nitrogen applied to the soil.

TABLE II

Effects of Sodium Cyanide and Nitrate of Soda on Growth Rate of Plants in Gloucester Loam. Results Expressed as Fresh Weight in Grams

Quantity (g.) and form of nitrogen used	Tobacco							Corn				
	Replicates						Total	Replicates				Total
0.16 g. $NaNO_3$	198	161	110	170	189	194	1022	138	185	180	174	677
0.32 " "	264	251	244	236	179	201	1375	202	222	208	225	857
0.48 " "	220	263	286	278	267	242	1556	219	270	286	218	993
0.16 g. NaCN	133	144	140	134	122	122	795	131	119	130	134	514
0.32 " "	185	176	176	156	166	168	1027	164	168	140	129	601
0.48 " "	176	186	157	189	146	197	1051	198	195	192	210	795
Control, no nitrogen	118	103	110	109	116	95	651	86	76	97	100	359
Difference between totals required for significance with odds greater than 19:1 =							167	With odds greater than 19:1 =				108

Three different amounts of nitrogen, namely, 0.8, 0.4, and 0.2 gram as sodium cyanide, ammonium sulphate, and nitrate of soda, singly and in combination were supplied to Gloucester loam. The results which accrued from the tests in which corn was employed as the growth indicator are assembled in Table III. Sodium cyanide in the group of cultures which received the largest increment of nitrogen was less effective in the promotion of the growth of corn than were ammonium sulphate and a mixture of

TABLE III

Relative Efficiency of Nitrogen Carriers. Growth Indicator, Corn. Results Expressed as Dry Weight in Grams

Quantity (g.) and form of nitrogen used	Yield per culture			Total
0.8 g. NaCN	70	64	60	194
0.8 g. $(NH_4)_2SO_4$	88	79	86	253
0.8 g. $NaNO_3$	63	71	67	201
0.8 g., equal amounts of $NaCN + (NH_4)_2SO_4$	52	70	57	179
0.8 g., equal amounts of $NaCN + NaNO_3$	61	62	64	187
0.8 g., equal amounts of $NaCN + (NH_4)_2SO_4 + NaNO_3$	74	77	66	217
0.4 g. NaCN	51	52	50	153
0.4 g. $(NH_4)_2SO_4$	71	71	62	204
0.4 g. $NaNO_3$	70	72	62	204
0.4 g., equal amounts of $NaCN + (NH_4)_2SO_4$	63	60	67	190
0.4 g., equal amounts of $NaCN + NaNO_3$	61	64	56	181
0.4 g., equal amounts of $NaCN + (NH_4)_2SO_4 + NaNO_3$	62	62	63	187
0.2 g. NaCN	54	59	49	162
0.2 g. $(NH_4)_2SO_4$	62	63	57	182
0.2 g. $NaNO_3$	69	61	57	187
0.2 g., equal amounts of $NaCN + (NH_4)_2SO_4$	56	57	60	173
0.2 g., equal amounts of $NaCN + NaNO_3$	52	55	53	160
0.2 g., equal amounts of $NaCN + (NH_4)_2SO_4 + NaNO_3$	55	60	54	169
Control, no nitrogen	53	43	53	149
Difference between totals required for significance with odds greater than 19:1 =				22

the different nitrogen carriers. The yields derived from the remainder of this group did not vary significantly.

The cultures to which 0.4 gram of nitrogen was applied as sodium cyanide afforded less growth of corn than did the remainder of the cultures. The combination which consisted of equal parts of nitrogen as sodium cyanide and sodium nitrate was next to the lowest in efficiency. Increases in yield were obtained from the culture which received 0.2 gram of nitrogen as nitrate of soda, ammonium sulphate, and as a mixture of sodium cyanide and ammonium sulphate.

Lettuce was employed as the test plant in a series of cultures identical to the one in which corn was grown, with the exception that the amounts of nitrogen were less. The results derived from this series of tests are presented in Table IV. Each treatment, included in the group to which 0.6 gram of nitrogen was added, resulted in greater yields than did the use of sodium

TABLE IV

RELATIVE EFFICIENCY OF NITROGEN CARRIERS. GROWTH INDICATOR, LETTUCE. RESULTS EXPRESSED AS FRESH WEIGHT IN GRAMS

Quantity (g.) and form of nitrogen used	Yield per culture				Total
0.6 g. NaCN	23	20	17	23	83
0.6 g. $(NH_4)_2SO_4$	30	28	32	34	124
0.6 g. $NaNO_3$	31	26	26	27	110
0.6 g., equal amounts of $NaCN+(NH_4)_2SO_4$	40	35	38	33	146
0.6 g., equal amounts of $NaCN+NaNO_3$	22	22	30	32	106
0.6 g., equal amounts of $NaCN+(NH_4)_2SO_4+NaNO_3$	34	47	36	40	157
0.3 g. NaCN	36	38	45	41	160
0.3 g. $(NH_4)_2SO_4$	40	34	38	35	147
0.3 g. $NaNO_3$	37	44	40	39	160
0.3 g., equal amounts of $NaCN+(NH_4)_2SO_4$	42	38	37	48	165
0.3 g., equal amounts of $NaCN+NaNO_3$	40	37	37	48	162
0.3 g., equal amounts of $NaCN+(NH_4)_2SO_4+NaNO_3$	51	51	42	50	194
0.15 g. NaCN	45	47	43	50	185
0.15 g. $(NH_4)_2SO_4$	45	47	44	49	185
0.15 g. $NaNO_3$	50	40	44	55	189
0.15 g., equal amounts of $NaCN+(NH_4)_2SO_4$	41	46	49	45	181
0.15 g., equal amounts of $NaCN+NaNO_3$	40	46	42	51	179
0.15 g., equal amounts of $NaCN+(NH_4)_2SO_4+NaNO_3$	42	50	41	40	173
Control, no nitrogen	20	18	24	17	79
Difference between totals required for significance with odds greater than 19:1 =					18

cyanide alone. It was observed that the growth rate was retarded about two weeks by the application of 0.6 gram of nitrogen as cyanide. The largest harvests were derived from the cultures which received the nitrogen in equal amounts from each carrier. The difference between the yields of lettuce derived from the group of cultures to which 0.3 gram of nitrogen was applied was not striking. An equal amount of nitrogen from each carrier also proved to be the most successful. Where 0.15 gram of nitrogen was incorporated with the soil, sodium cyanide proved to be as effective

as did either ammonium sulphate, nitrate of soda, or as the combination in which each carrier was included.

The residual effects from sodium cyanide were measured by growing white mustard in the above cultures (Table IV). The soil was removed from each container, mixed, returned, and seed planted in it. According to the results which comprise Table V, the beneficial effects derived from sodium cyanide were less than were those which resulted from either ammonium sulphate or nitrate of soda. The sodium cyanide, being less effective than these salts, acted as a diluent and smaller yields resulted when it

TABLE V

Residual Effects of NaCN. Mustard Following Lettuce

Quantity (g.) and form of nitrogen used	Yield per culture				Total
0.6 g. NaCN	55	50	63	42	210
0.6 g. $(NH_4)_2SO_4$	91	98	124	117	430
0.6 g. $NaNO_3$	100	85	114	134	433
0.6 g., equal amounts of $NaCN+(NH_4)_2SO_4$	83	95	78	100	356
0.6 g., equal amounts of $NaCN+NaNO_3$	96	100	97	95	388
0.6 g., equal amounts of $NaCN+(NH_4)_2SO_4+NaNO_3$	86	96	84	115	381
0.3 g. NaCN	33	25	23	24	105
0.3 g. $(NH_4)_2SO_4$	73	78	95	70	316
0.3 g. $NaNO_3$	78	103	60	68	309
0.3 g., equal amounts of $NaCN+(NH_4)_2SO_4$	47	55	55	53	210
0.3 g., equal amounts of $NaCN+NaNO_3$	47	67	61	53	228
0.3 g., equal amounts of $NaCN+(NH_4)_2SO_4+NaNO_3$	44	57	55	53	209
0.15 g. NaCN	18	22	20	24	84
0.15 g. $(NH_4)_2SO_4$	31	27	34	37	129
0.15 g. $NaNO_3$	50	53	55	45	203
0.15 g., equal amounts of $NaCN+(NH_4)_2SO_4$	26	27	28	34	115
0.15 g., equal amounts of $NaCN+NaNO_3$	36	31	35	36	138
0.15 g., equal amounts of $NaCN+(NH_4)_2SO_4+NaNO_3$	42	40	55	36	173
Control, no nitrogen	20	19	25	18	82
Difference between totals required for significance with odds greater than 19:1 =					36.3

was combined with either one or both of them. Where 0.15 gram of nitrogen had been added as sodium cyanide to the cultures, the yield of lettuce was not greater than was that derived from the control cultures. In the remainder of the cultures the yields were significantly greater than were those from the cultures to which nitrogen was not applied.

White mustard was utilized as the growth indicator in tests to determine the effect of leaching of soil treated with sodium cyanide and also nitrate of soda. According to the data which appear in Table VI, the beneficial effects of sodium cyanide are much more resistant to leaching than are those which result when nitrate of soda is added to a soil. With the exception of the cultures through which 16 inches of distilled water were passed, the yields derived from the use of sodium cyanide were significantly greater than were those taken from the control cultures. The cultures which had received nitrate of soda and which were leached with 8 and 16 inches of

water, respectively, produced less growth than was obtained from the control cultures.

TABLE VI

EFFECT OF LEACHING SOIL TREATED WITH SODIUM CYANIDE AND NITRATE OF SODA. GROWTH INDICATOR, WHITE MUSTARD. RESULTS EXPRESSED AS FRESH WEIGHT IN GRAMS

Cultural treatment*	Yield per culture			Total
NaCN	26	30	32	88
NaCN leached 4 inches	27	16	19	62
NaCN " 8 "	27	34	18	79
NaCN " 16 "	20	23	15	58
$NaNO_3$	62	66	64	192
$NaNO_3$ leached 4 inches	11	17	15	43
$NaNO_3$ " 8 "	6	7	12	25
$NaNO_3$ " 16 "	5	6	7	18
$NaNO_3$+NaOH leached 4 inches	17	13	14	44
$NaNO_3$+NaOH " 8 "	12	13	13	38
$NaNO_3$+NaOH " 16 "	7	5	7	19
Control, no nitrogen	14	17	19	50

Difference between totals required for significance with odds greater than 19:1 = 16.5

* 0.75 gram of nitrogen applied in each treatment.

Field tests. The results derived from the tests with tobacco grown in rims filled with soil make up Table VII. It is notable that although each treatment of sodium cyanide increased the yield of tobacco plants significantly, with the exception of those to which the smallest amount of nitrogen was added, the plots which received nitrate of soda produced more tobacco than did those which received nitrogen in the form of cyanide. The early growth rate of the plants receiving sodium cyanide was slower than that of the plants in the soil to which nitrate of soda had been added. During about the last six weeks of the growing period, however, the growth rate of the former increased greatly as indicated by the elongation and the dark green color of the plants.

TABLE VII

FERTILIZER VALUE OF SODIUM CYANIDE—FIELD TEST. GROWTH INDICATOR, TOBACCO. RESULTS EXPRESSED AS DRY WEIGHT IN GRAMS

Cultural treatment, lbs. nitrogen per acre	Replicates			Total
14.3 NaCN	60	51	56	167
28.6 "	75	79	81	235
57.2 "	86	88	94	268
14.3 $NaNO_3$	68	62	71	201
28.6 "	120	112	105	337
57.2 "	137	131	102	370
Control	30	35	32	97

Difference between totals required for significance with odds greater than 19:1 = 42

SUMMARY AND CONCLUSIONS

Sodium cyanide delayed for a few days the germination of radish, corn, and snap bean seed. It was more active in this respect when added to Leon sand than it was when mixed with Gloucester loam.

Sodium cyanide increased the yield of tobacco significantly but to a less degree than did nitrate of soda.

Sodium cyanide, nitrate of soda, ammonium sulphate, alone and combinations of these, were added to Gloucester loam. In general, sodium cyanide was the least effective for the growth of corn.

The growth of lettuce was not increased by the presence of 0.6 gram of nitrogen as sodium cyanide, probably on account of the retardation of the early growth of the plants, but it was increased when this amount of nitrogen was added to the soil in combination with either ammonium sulphate or nitrate of soda. An intermediate amount of nitrogen, 0.3 gram per culture, as sodium cyanide, ammonium sulphate, nitrate of soda, and combinations of these increased the yield of this crop significantly.

In general, the residual effect of sodium cyanide on white mustard was less beneficial than was that from ammonium sulphate, nitrate of soda, and combinations of these with sodium cyanide.

The beneficial effects derived from sodium cyanide were more resistant to leaching than were those derived from nitrate of soda as measured by the growth rate of white mustard.

In field tests sodium cyanide increased the yield of tobacco but to a less extent than did nitrate of soda.

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IMPORTANCE OF OXYGEN SUPPLY IN SECONDARY DORMANCY AND ITS RELATION TO THE INHIBITING MECHANISM REGULATING DORMANCY

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Dormancy of seeds manifested by a delay in germination when held under favorable germinating conditions is a common occurrence in nature. Crocker (3, p. 105) has described the mechanism by which seeds may be delayed in germination as follows: (a) Rudimentary embryos that must mature before germination can begin; (b) complete inhibition of water absorption; (c) mechanical resistance to the expansion of the embryo and seed contents by enclosing structures; (d) encasing structures interfering with oxygen absorption by the embryo and perhaps carbon dioxide elimination from it, resulting in the limitation of the processes dependent upon these; (e) a state of dormancy in the embryo itself or some organ of it, in consequence of which it is unable to grow with full vigor but may show great sluggishness in development when naked and supplied with all ordinary germinative conditions; (f) combinations of two or more of these; (g) assumption of secondary dormancy. By proper treatment or by a period of after-ripening the dormant seeds displaying these general characteristics will germinate and develop a normal plant. As Crocker has pointed out, there are conditions that will bring about a renewal of the dormancy at the time many seeds should be germinating so that the embryo will not develop until this induced resting stage is overcome by some general or specific upset to the mechanism involved whereby the embryo is released for growth. This induced dormancy is generally known as secondary dormancy and is not dependent upon a primarily dormant condition for its inception. That is, some seeds that normally show no dormancy, as well as others that have a normal dormancy within the embryo, may be made dormant by exposure to adverse conditions while being held, fully imbibed with water, on moist substratum.

It is thought that the process of secondary dormancy to be discussed in this paper is widespread in its operation in nature in increasing the life span of seeds. And it is the object of this paper to present some of the pertinent literature and to offer some facts and theories regarding the development, the continuation, and the breaking of secondary dormancy in plant organs.

Secondary dormancy lies in the embryo. This may be as a result of external germinating conditions being so unfavorable in bringing about a

limitation of the oxygen supply to the tissue as to have altered the metabolism as well as the chemical makeup of the embryo so that it is unable to function or to develop normally. The abnormal development persists for a considerable period of time even after the restraining conditions are removed. The nature of this process is not fully understood, but with *Brassica alba* the presence of carbon dioxide, which apparently reduces and also alters the normal respiration, will bring about changes in the metabolism of the embryo resulting in secondary dormancy. Kidd and West (15) believe that oxygen is necessary for this phenomenon although they reported that the best conditions for the development of secondary dormancy lie within a rather narrow range of concentrations of oxygen and carbon dioxide that will inhibit germination without causing irreparable injury to the embryo. This, however, need not be construed to mean that this is the ideal condition for developing secondary dormancy in all plants. It is to be expected with biological material that not all species will react alike; not all will require the same amount of oxygen or of carbon dioxide in pursuance of their metabolic activity. Kidd and West found that the removal of carbon dioxide from the external or internal atmosphere of the seed did not alter the period of dormancy. And they unexpectedly found that the seed coat remained permeable to oxygen and carbon dioxide both before and during the period of secondary dormancy. From their investigation it appears that the dormancy changes are completely within the embryo and that seed coats may exert some restraint only upon the expansion of the embryo through its intake of water. As long as the seed coat is intact the seeds will lie dormant under good germinating conditions for a year or more. When the coats are removed or cut in any manner, swelling takes place and the dormant seeds initiate germination within a few hours to a few days. Usually these seeds will develop abnormally; the cotyledons enlarge and become green preceding plumule and radicle growth in just the same manner as observed with induced secondarily dormant cocklebur seeds (5, 35), primarily dormant *Ambrosia trifida* seeds (4), and many other dormant seeds (11), when the coats are removed and the seeds are placed under germinating conditions.

Drying of the secondarily dormant *Brassica alba* seeds with coats intact will likewise break the dormancy so that the seeds will readily germinate when again moistened. This is a condition akin to primary dormancy in *Ambrosia trifida* that was found by Davis (4) to disappear to a considerable extent during dry storage. According to Kidd and West, this process of developing, then breaking, the secondary dormancy of *Brassica alba* can be repeated many times without causing permanent injury to the embryo.

Crocker (2) in 1906 reported that the delay in germination of the upper seed of the cocklebur, *Xanthium canadense*, was due to seed coat restricting

the supply of oxygen to the embryo and he suggested that this was the main reason for the delay in germination of these seeds in nature, since they displayed no dormancy if the seed coat was removed. Later Davis (5) was able to induce dormancy in the cocklebur embryo by embedding the moist seeds in clay or burying in agar for two months at 27° to 30° C. under which conditions presumably a low supply of oxygen was available to the seeds. The dormancy was subsequently overcome by removal of the seed coats or moist storage at 5° C. for three months. Davis found in nature that the embryos of the upper seed became dormant during the summer and after-ripened during the winter until the seed coat disintegrated sufficiently to admit the necessary amount of oxygen needed for germination. Besides this, Davis showed that the embryos of *Ambrosia trifida* which have a natural dormancy could be after-ripened in the usual manner, then buried in agar before they sprouted where they would develop a secondary dormancy having all the characteristics of the primary dormancy. This secondary dormancy could then be broken by after-ripening at 5° C. for three months. Davis observed also that the primary dormancy of *Ambrosia trifida* could not be broken by moist storage at 5° C. if the seeds were held in an atmosphere completely devoid of oxygen. These seeds maintained their primary dormancy as intensely as seeds stored dry at higher temperatures.

The work of Davis on the cocklebur was repeated by Thornton (35) who used controlled gas mixtures of O_2, CO_2, N_2, or H_2 with the result that secondary dormancy was induced in the moist seeds only when oxygen was absent from the atmosphere. The gas treatment of the seeds was carried on for five or more weeks at a temperature of 21° to 30° C., at which time the secondary dormancy was found sufficiently complete so that the embryos could be removed from the sealed flasks, placed on moist cotton in a Petri dish, and exposed to the normal content of oxygen in the atmosphere. If kept moist and with coats intact these seeds maintained their dormancy for a period of more than two years. Instead of the dormancy becoming less with storage in air, the dormancy became more deeply seated so that an even greater delay in germination was found upon removal of the seed coats. Upon removal of the coats the embryos displayed very slow growth by the enlargement and greening of the cotyledons and development of the plumules before that of the radicle. This growth always took place very slowly, requiring sometimes as much as five weeks to develop to a stage where the embryo could be transferred to soil. These are typical characteristics of the development of normally dormant and non-after-ripened embryos of many other species of seeds. When planted in soil these plants grew very slowly, with curly, deep green leaves and required approximately five weeks to readjust their metabolism in order that the normal type and rate of growth could take place. This secondary

dormancy could also be broken at the end of the gas storage by holding moist at 5° C. for three months after which the embryos germinated rapidly and developed normally.

The work of Crocker on Johnson grass, as reported by Harrington (12, 13), indicates that with the proper temperature and the right type of seed coat one can force into secondary dormancy a seed that normally displays no dormancy in the embryo. The coat of Johnson grass seed is highly suberized, compact, and relatively impervious to solutes and from this it is reasonable to expect that it is likewise relatively impervious to oxygen which leads to the development of secondary dormancy. Once dormancy was established it was readily broken by exposure to various concentrations of carbon dioxide and other chemicals.

Lettuce (*Lactuca sativa*) seeds, if held in a moist condition at a temperature of 35° C., do not germinate but will develop dormancy as shown by the fact that a delay in germination results if the seeds are removed to a more favorable temperature for germination. The period of this delay depends entirely upon the length of storage at the unfavorable temperature. Borthwick and Robbins (1) reported that this effect could be overcome by exposure of these seeds while at 35° C. to increased percentages of oxygen, but Thornton (36) reported that this was not the solution to the problem with seeds of six other varieties of lettuce. Thornton did find, however, that if the seeds were exposed to 40 to 80 per cent of carbon dioxide with 20 per cent of O_2 they would germinate readily and in a normal manner while held at 35° C. Seeds that had been held dormant for as much as three months at 35° C. were readily germinated in one week by this special gas treatment. Likewise, recently harvested lettuce seed that would not germinate in normal air at any temperature between 22° and 30° C. (though not developing dormancy) could readily be forced to germinate by treatment with from 5 to 80 per cent of carbon dioxide in the presence of 20 per cent of oxygen.

According to Thornton (34), carbon dioxide mixed with oxygen (40 to 60 per cent CO_2 and 20 per cent O_2) will bring about the sprouting of dormant potatoes within 16 to 20 days as compared to more than 60 days for the controls. No doubt, here again the carbon dioxide so alters metabolism that the mechanism of sprouting is set free since it has been found that the tubers show increases in reducing sugars, sucrose, catalase activity, conductivity, and the action of reducing substances. However, in this case it is not conceivable that any of these changes are the cause of breaking the dormancy since Miller, Guthrie, and Denny (21) have carried out exhaustive studies of this nature without finding any one of a number of changes in metabolism of the dormant tuber to be the causal factor in the breaking of its dormancy by chemical treatments. Of course, the potato cannot be strictly compared with a seed, yet the fundamentals of growth

are present although the mechanism by which it is accomplished may be different in each organ. And in view of this the information regarding the effects of many chemicals upon the dormancy and metabolism of the potato cannot be used in discussing the dormancy of seeds, mainly because it is apparent that the same chemical system or systems are not operative since usual chemicals, except CO_2, effective on potatoes have so far been observed to have little effect upon the dormancy of seeds. One exception to this is lettuce seed where the seed coat is apparently responsible for the observed dormancy. Thompson and Kosar (32, 33) have found that lettuce seeds dormant in the dark at 25° C. can be forced into germination by treatment with 0.5 per cent solution of thiourea. Thompson (31) has lately reported that these dormant seeds can be treated, dried, then subsequently germinated at 33° to 35° C. However, no reports have been made on the effect this chemical would have upon the germination of the seeds made dormant by holding moist at too high a temperature for germination (35° C.) in either light or darkness. In addition to the general idea that dormancy-breaking chemicals do not operate upon seeds with dormant embryos, it has been reported by Thornton (37) that high percentages of oxygen maintain the dormancy of the potato, and, contrary to the commonly accepted ideas, low concentrations of oxygen are responsible for breaking the dormancy of potatoes. Thus, it is interesting that a low percentage of oxygen should inhibit oxidation in the cocklebur (5, 35) and *Ambrosia trifida* (4) and produce a dormant condition, while in the potato it should also inhibit oxidation yet hasten the germination of the buds, thus indicating that we are working with two fundamentally different systems. If this is true then in various seeds themselves we have a different system or perhaps many systems operating with oxygen as one of the controlling factors. Morinaga (23) found that the germination of cat-tail seeds was markedly benefited by holding in atmospheres of 0.01 normal oxygen pressure contrary to the usual evidence that the germination of most seeds would be inhibited by such treatment.

In contrast to the effect of oxygen upon the buds of the potato, the buds of lily bulbs like some seed embryos will readily become dormant if the supply of oxygen is limited. Thornton (38) demonstrated by gas storage methods that *Lilium longiflorum* bulbs can be made dormant so that the bulbs will either not grow or that they will produce dwarf plants with the characteristic deep green color and short internodes. Since these bulbs react for the most part like the seeds that are stored in the absence of oxygen they would no doubt serve as a good source of large quantities of materials for a chemical investigation into this problem of induced or secondary dormancy.

This briefly is a review of some of the pertinent literature dealing with the effect of oxygen upon the dormancy of plant organs, especially of

seeds. Although our knowledge of the facts as to the operating mechanism of secondary dormancy is far from complete, one can consider theoretically the possible ways by which this phenomenon could be brought about. Wollny (41), Delassus (6, 7), and later Urbain (40) studied the effect of reducing the food supply of seeds upon the subsequent growth of the embryos and plants. In general, they determined that by cutting the cotyledons and thus reducing the food supply (including possibly essential growth substances) to the embryo, the resulting growth was slow and greatly reduced under that of the plants grown with intact cotyledons. These investigators also observed that the plants grown with mutilated cotyledons were dwarfs with short internodes and that the plants failed to produce for some time secondary root systems. This is analogous to the condition found in plants from secondarily dormant cocklebur seeds and in the plants produced from lily bulbs made partially dormant by storage without oxygen. Is it possible that the lack of oxygen acts upon the storage mechanism just the same as if the plant is deprived of part of its stored food?

Although it is believed that oxygen supply is the controlling factor in secondary dormancy it cannot and should not be regarded as the single causal factor in the development of secondary dormancy in plant organs. Instead, oxygen supply must be considered in conjunction with all other factors, temperature, seed coat or other protective tissue, moisture supply, chemical composition, etc., that are effective in controlling the general metabolic activity of living tissue. With this viewpoint in mind, it is of interest to consider some of the information that is available regarding the chemical changes taking place in seeds held under various conditions.

Maze (20) in 1900, while investigating the effect of soaking seeds, concluded that seeds placed under water may preserve for some time a condition of latent life, but at the same time that the hydrolyzing enzymes maintain their activity, the oxidizing enzymes are unable to perform their normal function to supply the embryo with food material and to remove intermediary products. Under this condition starchy seeds lose their vitality rapidly while the oily seeds retain their vitality for some time. Maze believed that the continued accumulation of acetaldehyde in the absence of an opportunity for its oxidation eventually brought about the loss of vitality of the seeds and it is conceivable that an analogous situation occurs when the seeds are held under germinating conditions where an insufficient supply of oxygen is available to the embryo.

Eckerson[1] has found that when wheat seeds are germinated at 24° C. the proteins break down rapidly to polypeptids and thence very slowly to amino acids; at the same time there is a slow hydrolysis of starch to sugars.

[1] Private communication.

The embryo is surrounded by a mucilaginous substance (which no doubt retards the entrance of oxygen) with the result that it is slow to develop and a small, weak plant is formed. However, at low temperature, 10° C. or below, starch is hydrolyzed rapidly to sugars and the proteins are broken down slowly to amino acids without any detectable accumulation of polypeptids which is the normal order in the metabolism of the wheat seeds. The resulting seedling growth is rapid, and a sturdy plant is produced that is relatively resistant to fungous invasion.

Eckerson[2] has also determined the presence of acetaldehyde in normally dormant seeds and has observed its disappearance as dormancy is broken and growth is initiated. She has reported (9) that with the breaking of dormancy in *Crataegus* by low temperature storage the embryo and surrounding tissue change from a neutral or slightly alkaline reaction to an acid condition at which time the enzyme system becomes active and growth is initiated. From these results it is possible that the oxidizing system may be inhibited thus accounting for the accumulation of acetaldehyde and the primary dormancy. With prolonged storage at low temperature the inhibiting substances are finally eliminated by a process of slow oxidation whereby such substances as acetaldehyde are converted to acids and on to various end products so that growth is permitted and at the same time aided in getting under way. It is significant that Eckerson found that the chemical changes taking place during 90 days of after-ripening of dormant *Crataegus* seeds at 5° C. were the same as those taking place in non-dormant *Ricinus* seeds during the first eight days of germination and that dormant seeds of *Pyrus baccata*, which after-ripen in 30 days, had, at the start of moist storage at 5° C., a similar internal chemical relationship to that in *Crataegus* seeds after 60 days' storage at 5° C. Pack (26, 27) in a chemical and physiological study of after-ripening and germination of *Juniperus* seeds showed the multiplicity of changes that accompany after-ripening at 5° C. With respiration retarded, the combustion of materials was reduced to a minimum, thereby favoring the accumulation of formative materials in the cells exposed to a normal supply of oxygen with the result that dormancy was overcome.

Harrington (14) determined that starch and cane sugar were hydrolyzed more rapidly than they were respired with the result that there was an accumulation of reducing sugars during the development of secondary dormancy in Johnson grass seed held under germinating conditions at 20° C. If the seed coat was removed this seed would readily germinate when first placed in the germinator instead of becoming dormant; thus it is evident that the coat must be a factor in altering the metabolism of this seed by reducing the supply of oxygen to the embryo at a time when it is

[2] Private communication.

greatly needed. Harrington reported that the closely related Sudan grass seed germinated rapidly if held under the same conditions that would make the Johnson grass seed dormant. He observed that the seed coat structure of the Johnson grass was much more compact, with considerable suberized tissue, and was relatively more impermeable to solutes than the seed coats of the Sudan grass. In view of these findings it is reasonable to conclude that the fundamental difference in reaction of these two seeds to germinating conditions at 20° C. is due to the relative impermeability of the coat of the Johnson grass to oxygen with the result that dormancy is induced while oxygen penetrates the seed coat of Sudan grass permitting it to germinate readily.

Thus it appears that secondary dormancy may be the direct result of an accumulation of inhibiting substances (developed during partial anaerobic respiration when sufficient oxygen was not available to the tissues) in such quantities as to partially poison the oxidizing system. Even when oxygen becomes readily available (by removal of the seed coat) at favorable germinating temperatures, the system can function only slowly and partially, thus accounting for the slow recovery of the embryo to a normal condition. With the intake of a high percentage of water when the seed coat is not present to retard swelling (which the coat can accomplish since there is no growth process to rupture it), the intermediate products or inhibiting substances may be diluted and leached out, thus rendered less toxic to the embryo. This, together with a return of the system to a predominance of aerobic metabolism, permits the embryo to begin its recovery to normal. With small seeds (lettuce and mustard) this could be relatively rapid while with larger seeds (cocklebur and *Ambrosia*) this process would be very slow.

Besides the expected alteration in the chemical changes involved in living tissue placed under partial anaerobic conditions, there is also the effect upon chromosome reproduction and cell division. Nabokich (24) reported that lack of oxygen caused complete cessation of mitotic activity in pea, bean, and sunflower. More recently, Steinitz (29) has shown that an anaerobic condition causes many cytological abnormalities in both root tip and shoot meristem tissue of barley. Clumping of chromosomes, sticky anaphase bridges, and extrusion of chromatic material into the cytoplasm were observed. When the recovering plants were held in the greenhouse, no gross morphological abnormalities were obtained except some twisting of the leaves, which could be important if this twisting was akin to the curling of leaves observed in the production of plants from dormant embryos. In other studies on meiosis in *Tradescantia*, Steinitz (30) found despiralized metaphases where the tissue was held in absence of oxygen at 28° C. Steinitz considered that the connection between the lack of oxygen and the altered organization of the cell may be the result of the develop-

ment of products that poison the cell together with insufficient production of sterols thought necessary to maintain the surface tension of the interface between chromosome and cytoplasm. These observations also suggest the possibility of chromosome abnormalities and inhibitors being produced in seeds thrown into secondary dormancy while held moist at high temperatures under conditions of insufficient supply of oxygen.

Maintaining the seed coat intact serves to limit the exchange of oxygen (and other gases and volatile products); thus the seed exists under partially anaerobic conditions which favor the development of intermediary products detrimental to normal germination. Even though the metabolic processes are greatly reduced at this stage, continued maintenance of the seed coat intact serves to keep these products concentrated and in position to exert their greatest inhibitory action, and therefore oxidizable only slowly although the potential oxygen supply may be far above that normally required by the tissue. For example, the lower intact seed of the cocklebur requires only 6 per cent of oxygen in the atmosphere to initiate germination under normal conditions, but if the seed has been exposed to an atmosphere devoid of oxygen the seed will not germinate but instead will remain dormant. If, however, the coat is removed the seed will then slowly develop in an abnormal manner (enlargement of cotyledons and growth of the plumule preceding the growth of the radicle) and will require at least five weeks to produce a normal leaf and to grow at a normal rate.

While storage at high temperatures seems to increase the relative rate of oxidation, it also increases the rate and affects the nature of hydrolysis of stored materials so that there is a continuance of the excess of inhibiting substances and therefore a continuance of dormancy. As already mentioned in the case of the cocklebur seeds, the induced dormancy becomes much more intense when the moist-held seeds are removed from the oxygen-free atmosphere to one containing a normal supply of oxygen at a temperature of 20° to 30° C. With moist storage at low temperature, 5° C., the dormancy is broken because the hydrolyzing system is more orderly and its products altered; the oxidation system too can slowly regain its normal activity since only a small portion of the oxygen taken up is needed to support actual end-product respiration (oxidation of the simple carbohydrates), leaving a larger portion available to complete basic chemical changes in bringing about the elimination of those substances causing the dormant condition.

The presence of inhibiting substances in plants and more specifically in seeds has been of interest to physiologists for many years. Evenari (10) has reviewed the subject of germination inhibitors bringing together a large number of theories on the subject. Oppenheimer (25) reported on inhibiting substances present in tomato and many other fruits, and sub-

sequent workers have many times substantiated these results. Molisch (22) in 1937 and later Konis (18) concluded from their experiments that probably ethylene was the inhibitor in these tissues since Denny and Miller (8) in 1935 had reported ethylene given off by tomato fruits. Sroelov (28) reported the presence of inhibiting substances in dry fruits, and Ullman (39) found essential oils, alkaloids, and glucosides efficient inhibitors of the germination of seeds. According to Ullman, bitter almonds containing amygdalin in large quantity will inhibit germination of wheat and mustard seeds much more effectively than sweet almonds which contain a very low content of this glucoside. Since HCN is known to inhibit germination of seeds in very low concentration [1 to 10,000 according to Ullman (39)], its presence in seeds leads one to assume that it is one of the effective agents in inhibiting germination. Laibach and Keil (19) and later Keil (17) studied the HCN produced when seed paste, containing amygdalin and emulsin, was placed near tomato seeds under germination conditions. Keil believes that HCN not only retards germination in some species, but also its presence is necessary for best germination of apple seeds, thus acting both as a poison and as a stimulant. Eckerson (9) found in her studies of *Crataegus* seeds that HCN was liberated from the seeds at the latter part of the after-ripening period just previous to germination of the seed. This observation may indicate that the amygdalin, usually hydrolyzed very slowly, was broken down very rapidly toward the end of the period of after-ripening, the HCN liberated from the tissue, then germination processes once poisoned by the HCN were freed to function in a normal manner.

The results of many individual reports indicate that almost every seed has a specific range of temperature at which secondary dormancy may be developed. The seed coat plays an important role in this process and the nature of its structure may determine the temperature required to initiate secondary dormancy. For example, Johnson grass seed with a highly suberized coat will become dormant at 20° C. while lettuce seed with a relatively thin coat requires a temperature of 35° C. and cocklebur seed also with a relatively thin coat plus a very low oxygen requirement for general metabolism requires mainly an exceedingly low oxygen supply and a temperature intermediate between these extremes before it can be made dormant. The important factor in these cases, of course, is the rate of passage of oxygen through the coats since usually with each increase in temperature more oxygen is required to meet the metabolic needs of the embryo.

Finally a point is reached where the available supply of oxygen is insufficient for the oxidation of all the products of hydrolysis as fast as they are formed and then secondary dormancy has its inception. This immediately suggests one of the important phases of this problem that should

receive a great deal of attention and that is the permeability of seed coats to oxygen and other gases and the results of differences and changes in permeability during development upon the chemical composition of the embryo. Were one able to answer some of the problems involved in the development and elimination of dormancy he would have, no doubt, a better insight into the growth of embryos.

Primary and secondary dormancy appear to be identical since seeds with or without the former can be thrown into the latter repeatedly and at intervals of variable duration over a long period of time. From this result it is evident that primary dormancy is brought about by the lack of oxygen supply to the developing embryo. With apple, rose, and many other seeds the oxygen demand of the tissue surrounding the seed embryos may be so great during development that the embryo exists on an insufficient supply, thus the oxidizing system is greatly inhibited, toxic substances accumulate, and dormancy of varying depth is the result. The exact nature of the dormancy may also be regulated by the type of stored substances within the cotyledons since it is not to be expected that the same substance or substances will influence embryo development in the same manner in all species of plant life. Likewise, the depth of dormancy will vary with every species and with the conditions under which they are developed. The developing seed of pea or mustard studied by Kidd and West (16) was found to be delayed in germination when removed from the plant in a green ripe stage. This condition, though temporary and readily eliminated by drying of the seed or removing the seed coat, was thought to have been brought about by the lack of oxygen penetration through the layer of moisture separating the young embryo from the green seed coat. In this case the embryo of the pea was probably exposed to considerably more oxygen during its development than was the embryo of the apple (which is cut off from oxygen supply even from the opening at the flower end), not only because of the shorter period of development, but also because the pea embryo is not surrounded by an enormous amount of storage tissue like that in the apple. This would account for the short period, and in fact, almost no dormancy in the pea as compared with the greater depth of dormancy in the apple.

In conclusion, this problem of dormancy is a fertile field for research, having to do with the permeability of plant membranes to gases and with chemical changes within the tissues during the onset and elimination of dormancy. At this time when abnormal growth of living cells continues to baffle research investigation, the effect of oxygen and also of carbon dioxide upon the chemical and physiological changes in seeds (either directly or indirectly) which result in normal or abnormal development is not only of interest but is of fundamental importance.

SUMMARY

Secondary dormancy, and even primary dormancy, has its inception, it is believed, in the accumulation of intermediate products, formed by partial anaerobic respiration, that act as inhibitors because the oxidation system has been temporarily impaired through an insufficient supply of oxygen. The system is therefore unable to function in a normal manner in the removal of many products, among which may be mentioned acetaldehyde, reducing sugar, polypeptids, and no doubt many other substances in quantities so small that no attempt has been made to measure them.

It is further believed that the structure of the seed coat, the extent and metabolic activity of tissue surrounding the developing seed, and the external factors such as temperature, moisture, carbon dioxide, so alter the amount of oxygen available to the embryonic tissue that any degree of depth of dormancy may develop in the seed. The dormancy may be either of short duration as in the newly harvested pea or wheat seed, or of longer duration as in the apple or pear seed, or it may be restricted to only one portion of the embryo as in the tree peony seed.

High temperature storage seems only to augment the dormant condition in the majority of cases because the hydrolyzing system remains active over and above the inhibited oxidation system while low temperature storage not only alters the type of hydrolysis but retards the accumulation of inhibiting substance so that the metabolic activity may regain its normal activity.

The removal of the seed coats tends to dilute the inhibiting substances by allowing a great increase in water absorption and thereby aids a slow return to normal as shown by the slow abnormal growth of the plant.

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THIOUREA (THIOCARBAMIDE): ADULT LIFE SPAN FEEDING EXPERIMENTS WITH RATS

ALBERT HARTZELL

The present paper is a continuation of the results obtained from feeding experiments to determine whether thiocarbamide, also called thiourea, NH_2CSNH_2, was at all toxic when used in quantities likely to be ingested in the consumption of food treated with this compound (6, 13).

As reported previously (14) mice fed thiourea throughout their adult life span for a period of 24 months in the drinking water at the rate of 1.72 mg., 6.88 mg., and 27.5 mg. per kg. of body weight daily, were normal in appearance and in growth response. Even when fed the highest amount of thiourea the mice showed no indication of cumulative effect, nor was there any evidence that thiourea either increased or diminished the mortality rate. The present paper gives the results obtained with the same series of rats as reported previously (14), but throughout their entire life span, or for approximately three years. In addition, short time experiments with rats with thiourea introduced in the food and injection tests with thiourea on normal and paratyphoid-infected rats are reported.

Flinn and Geary (7) found no unfavorable effects on rats and rabbits fed with 300 to 700 times the amount of thiourea that would be ingested by a man eating 200 g. of treated fruit.

Since the publication of the above mentioned paper, Richter and Clisby (20) found that phenyl thiourea produced thyroid enlargement and other toxic reactions when fed to rats. MacKenzie and MacKenzie (18) found that enlargement and hyperemia of the thyroid glands in rats, mice, and dogs were produced by feeding sulfaguanidine, sulfanilamide, sulfadiazine, sulfapyridine, thiourea, diethyl thiourea, and allyl thiourea. Guinea pigs and chickens gave a negative response as regards thyroid increase when fed sulfaguanidine. Administration of iodine in the diet did not prevent the thyroid changes while small amounts of thyroxin (1 μ gram per 10 grams of body weight) administered subcutaneously daily completely nullified the goitrogenic effect or even reduced the size of the gland below normal. The action of these drugs was very similar on both pituitary and thyroid to that described for animals fed a 45 per cent rape seed diet. Only one test was continued beyond 58 days. This test, however, indicated that equilibrium had already been established and that decrease of weight of the thyroid had begun, as was found in tests with rape seed reported by Griesbach (11) and others (12). The thyroid enlarging effect of the sulfonamides and thioureas is probably mediated through the pituitary as hypophysec-

tomy likewise prevents the thyroid alteration resulting from feeding 2 per cent sulfaguanidine to rats. Astwood, Sullivan, Bissell, and Tyslowitz (2) have substantially confirmed these results. Among the compounds tested by these authors were sulfadiazine, sulfapyridine, sulfathiazole, sulfanilylurea, sulfaguanidine, sulfanilamide, sulfasuxidine, and thiourea. The drugs were fed at various percentages of the diets or drinking water and by this means the authors were able to estimate with reasonable accuracy the relative effectiveness of the drugs in producing minimal and maximal thyroid effects. Sulfadiazine was the most effective sulfonamide tested. At a daily intake rate of 4.2 mg. per 100 g. of body weight it gave the minimal thyroid effect, while sulfaguanidine required 51 mg. per 100 g. of body weight for minimal effect. Thiourea exerted a minimal effect when only 3.3 mg. per 100 g. of body weight were given per day. In one case the tests extended for 68 days using 1.00 per cent of thiourea fed in the drinking water. A maximum degree of thyroid hyperplasia was produced by this feeding. The highest rate of feeding reported by Astwood *et al.* (2) for thiourea was 2 per cent of the food for 35 days. Beyond the effects on the pituitary and thyroid, they found no indication of deleterious effects.

Recently Astwood (1) has fed thiourea and thiouracil to man at a daily dosage of 1 to 2 g. Thiourea is stated to be about one-third as active as thiouracil when tested on rats. One to 2 g. daily of thiourea was found to be an adequate dose for initial therapy for man and about 0.5 g. daily for maintenance. It is believed that 1.5 g. daily of thiourea is too large for a maintenance dose in some cases as symptoms of myxedema and skin rash were produced by feeding at this level for five weeks in one of the goitrous cases. In the preliminary tests two normal persons were given 2 g. of thiourea daily for from 13 to 17 days. No change in basal metabolism was produced by these dosages, even when carried on for four weeks, and there was no observed thyroid change. Comparable dosages to men with hyperthyroidism lowered basal metabolism in 10 to 14 days. These differences are believed to be due to the storage of colloid in the normal thyroid gland and lack of this in the goitrous individual. Men with hyperthyroidism were greatly benefited with dosages comparable to the above due to the lowering of their metabolism, but when the dosage was discontinued the patients soon returned to their previous state of high metabolism.

FEEDING TESTS WITH RATS

Because of the difficulty of feeding a large number of animals by means of a stomach tube or pipette, in the present investigation thiourea was dissolved in tap water and fed in the drinking water. Albino rats[1] were selected for the feeding tests. Previous experiments had indicated that the

[1] Sherman strain obtained from Columbia University, New York, N. Y.

rat would drink approximately 29.2 cc. per day under normal conditions of temperature. The weight of the rats at the beginning of the experiment averaged 161.9 g. One hundred nineteen female rats, approximately three months old, five to a cage (except one cage with four rats), were used in these tests as follows:

(a) 30 rats fed 1.72 mg. per kg. of body weight daily, or 20 times man's dose.[2]
(b) 29 rats fed 6.88 mg. per kg. of body weight daily, or 80 times man's dose.
(c) 30 rats fed 27.5 mg. per kg. of body weight daily, or 320 times man's dose.
(d) 30 control rats.

The ration consisted of complete Rockland rat pellets. Carrots or lettuce were fed once a week. Twice a week cod liver oil and dried yeast were supplied.

The rats were weighed by cage groups at 10-day intervals until full grown and the doses of thiourea (recrystallized three times) adjusted to the average gain or loss in weight. After the rats were full grown, weighings were made every two weeks and doses adjusted accordingly.

All rats in the experiment were tested bimonthly for the presence of paratyphoid bacilli in the feces by the brilliant green agar method,[3] the results of which have been negative.

In this study four criteria were used in making comparisons between controls and treated rats:

(a) General appearance and vigor.
(b) Average weight of the rats (by cage groups).
(c) Average increase in weight from one weighing period to another.
(d) Longevity.
(e) Gross pathologic changes as revealed at autopsy.

The general appearance and vigor of the rats were normal in both the controls and treated (Figs. 1 and 2). The effect of these treatments on the average weight of the rats is shown in Figure 3.

The mortality of the rats throughout the experiment is shown graphically in Figure 4.

The number of rats surviving at the end of the first year of the experiment was 29 for the highest concentration (27.5 mg. per kg.), 28 for the middle concentration (6.88 mg. per kg.), 27 for the lowest concentration (1.72 mg. per kg.), and 28 for the control. The number of rats surviving at the end of two years was 20 for the highest concentration, 24 for the middle

[2] Man's dose equals 0.086 mg. per kg. of body weight, which is the approximate amount a man would ingest in eating 200 g. of treated fruit tissue per day.

[3] We are indebted to Dr. C. A. Slanetz of Columbia University, New York, N. Y., for the paratyphoid tests.

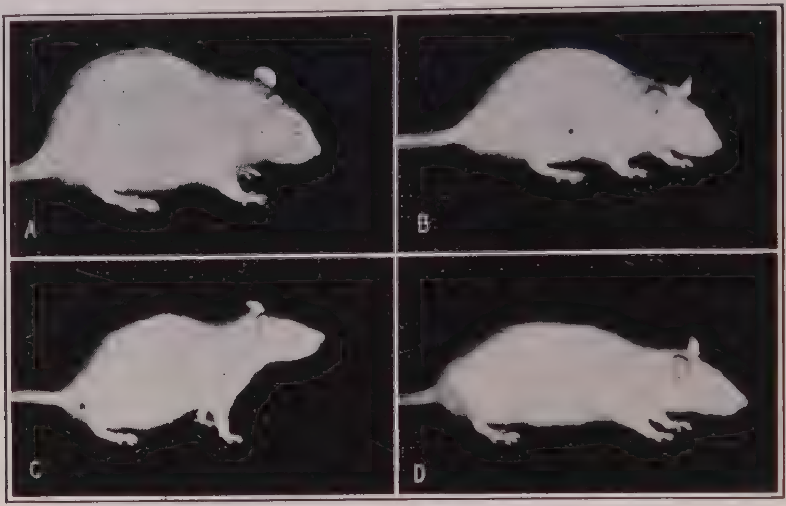

FIGURE 1. Rats used in thiourea adult life span feeding experiment photographed at the end of one year and six months (1/4 natural size). A. Control. B. Rat fed 1.72 mg. per kg. of body weight, or 20 times man's dose. C. Rat fed 6.88 mg. per kg. of body weight, or 80 times man's dose. D. Rat fed 27.5 mg. per kg. of body weight, or 320 times man's dose.

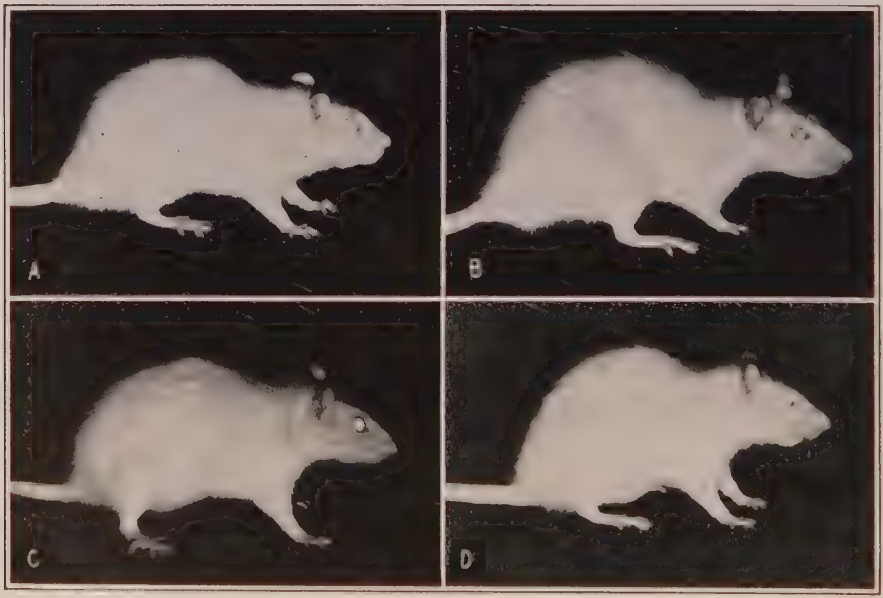

FIGURE 2. Rats used in thiourea adult life span feeding experiment at the end of two years (1/4 natural size). A. Control. B. Rat fed 1.72 mg. per kg. of body weight, or 20 times man's dose. C. Rat fed 6.88 mg. per kg. of body weight, or 80 times man's dose. D. Rat fed 27.5 mg. per kg. of body weight, or 320 times man's dose.

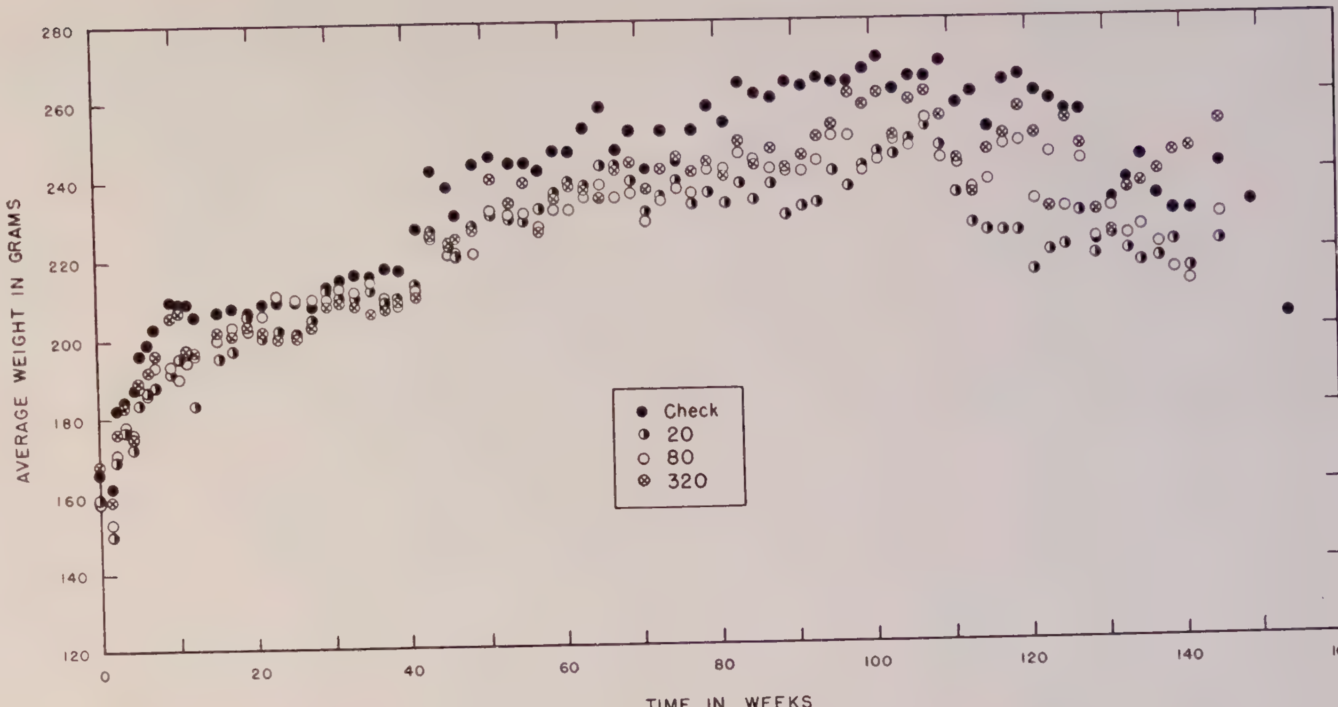

FIGURE 3. Curves for average weight of rats fed thiourea in adult life span feeding experiment in the following amounts in mg. per kg. of body weight: 1.72, or 20 times man's dose, including a corrected point (10 days) which erroneously appeared in the previously published chart (14); 6.88, or 80 times man's dose; and 27.5, or 320 times man's dose.

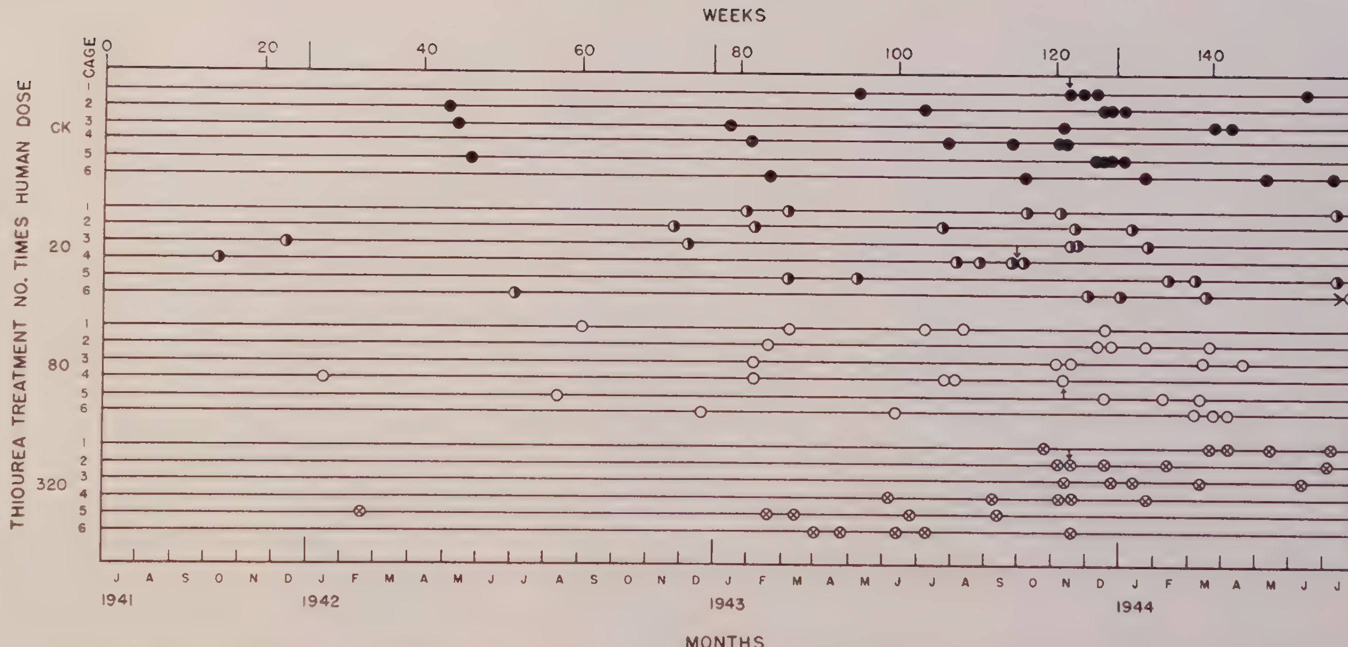

FIGURE 4. Mortality chart of rats fed thiourea in adult life span feeding experiment in the following amounts per kg. of body weight: 0 (check) = solid circle; 1.72, or 20 times man's dose = half solid circle; 6.88, or 80 times man's dose = open circle; and 27.5, or 320 times man's dose = × in circle. Last rat (1.72 mg./kg. concn.) died November 20, 1944, located on this chart with > sign in front of it. Fifty per cent mortality points indicated by vertical arrows.

concentration, 20 for the lowest concentration, and 23 for the control. At the end of the third year of the experiment two rats survived in the highest concentration, three in the lowest, and one in the control.

Deaths were due mainly to pneumonia, middle ear disease, and breast carcinoma. The rats which died of breast carcinoma were littermates which were later traced to a carcinomatous mother.

Autopsies made of rats that died showed no evidence of epidemic disease, nor pathologic change that could be attributed to thiourea (Table I). Tissues of representative organs were preserved in 10 per cent formalin solution and histological preparations were made, the results of which will be published later.

TABLE I

TYPICAL GROSS FINDINGS AT AUTOPSIES* OF RATS** WHICH DIED IN THE THIOUREA FEEDING EXPERIMENT

Organs	Concentration of thiourea times man's dose†			
	0 (Control)	20 (1.72 mg./kg.)	80 (6.88 mg./kg.)	320 (27.5 mg./kg.)
Brain	Normal	Normal	Normal	Normal
Thyroid	Normal	Not enlarged	Not enlarged	Not enlarged
Lungs	Bronchopneumonia and acute fibrinous pleuritis	Peribronchial foci of pneumonia, with mononuclear infiltration	Pneumonia; right, completely consolidated, left, emphysema	Right and left consolidated, pseudotuberculosis lesions
Heart	Normal	Normal	Normal	Normal
Spleen	Normal	Normal	Normal	Normal
Liver	Normal	Normal	Normal	Normal
Stomach	Normal	Normal	Normal	Normal
Intestine	Normal	Normal	Normal	Injected
Kidneys	Normal	Normal	Congested	Normal

* The writer is indebted to Dr. Ward H. Cook and Dr. Kendrick McCullough of the Bureau of Laboratories, Yonkers, N. Y., for autopsies of animals.
** Based on 34 individuals.
† Man's dose equals 0.086 mg. per kg. of body weight.

Astwood and others (2) have reported that it requires 3.3 mg. of thiourea per 100 g. of body weight of rat (33 mg. per kg.) fed in the drinking water to give a minimal effect in inducing thyroid hyperplasia. The maximum dose (27.5 mg. per kg.) in the present writer's experiments is below Astwood's minimal dose.

MacKenzie and MacKenzie (18) fed rats weighing 50 to 75 g. food containing 0.05 per cent thiourea and report a minimal increase in weight

of the thyroid glands at this concentration. The highest dose used in the present report appears to be at about the threshold of MacKenzie's minimal dose.

EFFECT ON WEIGHT OF YOUNG ADULTS

It will be observed in Figure 3 that the average gain in weight of rats fed thiourea fell off during the first ten days of the experiment. The fact

TABLE II

EFFECT ON WEIGHT OF TWO-MONTH-OLD RATS FED THIOUREA IN THE DIET FOR 44 DAYS

Sex	Amt. in g. of thiourea per kg.	Cage No.	Gain in g. per cage per day — In the intervals: Start to 44 days	14 to 44 days
Female	27.5	9	2.75	2.50
		3	2.86	1.80
		18	2.82	2.10
		13	2.89	1.56
		Av.	2.83	1.99
	6.88	5	3.23	3.23
		17	3.45	2.63
		16	4.41	3.03
		Av.	3.70	2.96
	Check	1	3.91	2.73
		11	4.82	3.57
		7	3.75	2.97
		15	5.23	4.00
		Av.	4.43	3.32
Male	27.5	8	7.02	5.87
		14	8.39	5.87
		Av.	7.70	5.87
	6.88	2	7.96	5.50
		10	6.68	5.50
		Av.	7.32	5.50
	Check	4	9.20	6.87
		6	9.07	7.07
		Av.	9.14	6.97

that the control also showed a parallel trend caused the writer to believe that this was due to some unfavorable environmental condition, possibly due to the relatively high temperatures during July 1941, when the experiment was begun.

It will be noted that in Figure 3 the control lots were consistently heavier than the other lots. This difference became apparent even in early

weeks of the test and was maintained until toward the end of the feeding period, the difference not increasing perceptibly, however, over the period from 40 to 120 weeks. This indicated that the early period of the feeding test was especially important, and possibly determinative for the balance of the experiment.

In order to test this further, at the close of the life span experiment a feeding test was set up using two-month-old rats fed thiourea in rat pellets

TABLE III

THIOUREA FEEDING AND INJECTION TESTS WITH FIVE-MONTH-OLD RATS. OBSERVATION OVER A PERIOD OF TWO WEEKS

Treatment	No. rats	Wt. range, g.
18 Mg. thiourea* per rat, orally	4	350 358 361 365
18 Mg. thiourea* per rat, intraperitoneally	4	266 269 300** 312
100 Mg. thiourea† per kg., intraperitoneally	4	270 275 283 300
1.5 Cc. N saline per rat, intraperitoneally	4	265 281 300 303
Control	3	250 265 300

* Dissolved in 1 cc. sterile water.
** Died 3 to 6 hours after injection.
† 27 to 30 mg. thiourea dissolved in 1.5 to 1.7 cc. sterile water.

at the same daily rate as in the previously mentioned life span experiment. This test was continued for 44 days. The results appear in Table II.

It will be observed from Table II that the rats fed the highest concentration of thiourea (27.5 mg. per kg.) gained less per day than the checks. This difference is significant at least for the females with odds of 100:1. The male rats fed 27.5 mg. per kg. and the rats of both sexes fed 6.88 mg. per kg. per day also gained less per day during the 44-day period than the checks, but the odds are less than 19:1, so the difference in this case may not be significant. These results are in general agreement with the previous test.

EFFECT OF THIOUREA ON THE MORTALITY OF TWO-MONTH-OLD AND FIVE-MONTH-OLD RATS

MacKenzie and MacKenzie (19) have reported that rats two months old are comparatively resistant to thiourea while five-month-old rats are highly susceptible to thiourea with mortalities ranging from 80 to 100 per cent, when injected intraperitoneally at a concentration of 100 mg. per kg. of body weight, or when fed at the rate of 18 mg. per rat (210–380 g. range). The present writer has attempted to repeat critical features of their tests using the same concentrations of thiourea and rats of the same age.

Two-month-old rats were injected intraperitoneally in groups of four each with 45 mg., 90 mg., and 180 mg. of thiourea. In addition, 125 mg. of thiourea were administered orally to each of two rats two months old. The controls consisted of four rats two months old each injected intraperitoneally with 2 cc. normal saline solution. All dilutions of thiourea and saline were made with sterile water. The average weight of the rats used in these tests was 124 g. There was no mortality among the rats in this series.

The results obtained with five-month-old rats appear in Table III. It will be observed from the table that only one five-month-old rat died in contrast to 80 to 100 per cent deaths reported by MacKenzie and MacKenzie (19) in their experiments. It is also of interest that none of the rats died to which thiourea had been administered orally in our test.

EFFECT OF THIOUREA ON RATS INFECTED WITH PARATYPHOID BACILLI

It was thought that the differences in results might be reconciled on the assumption that the rats used by MacKenzie and MacKenzie (19) may have been inadvertently infected with paratyphoid disease, a common malady of rats and one difficult to detect in the incipient stages except by bacteriological methods. Accordingly eight rats, five months old, distributed four to a cage, were infected with paratyphoid organisms[4] by feeding them 1 cc. each of a paratyphoid broth suspension on pieces of white bread. The feces of these individuals gave a positive reaction on brilliant green agar in five days. Four of these rats were injected intraperitoneally each with 36 mg. of thiourea while the four remaining rats were fed thiourea at the rate of 36 mg. per rat. A comparable series of healthy rats were used as controls, injected and fed thiourea in the same amounts to a like number of rats of the same age. There were no deaths among the healthy or the paratyphoid-infected rats (124 g. av. wt.) within two weeks, after which the rats infected with paratyphoid were sacrificed.

[4] *Salmonella enteritidis* Gärtner obtained from Dr. C. A. Slanetz of Columbia University, New York, N. Y.

DISCUSSION

It has been known for some time that substances containing certain organic sulphur compounds, particularly isothiocyanates, found in seeds, roots, and leaves of plants, especially rape, mustard, cabbage, and others of the genus *Brassica*, regularly used as food for animals, including man, produce enlargement of the thyroid gland, hyperplasia and loss of colloid, and hypertrophy of this organ through action on the pituitary (3, 16, 21, 15, 22). This action is not nullified by feeding iodine as is the case with certain foods but seems to be entirely inhibited by feeding thyroxin or thyroid powder (18). Hypophysectomized rats also do not show this effect. The action reaches an equilibrium point in about 56 days of feeding after which involution begins and colloid storage again takes place.

The following pure substances have been shown to have very similar effects on the thyroid and pituitary: sulfonamides (sulfathiazole, sulfapyridine, sulfadiazine, sulfaguanidine, etc.), thiourea, allyl thiourea, phenyl thiourea, and thiouracil. They range in effectiveness in the following order: thiouracil, thiourea, and sulfadiazine (2).

It is known that thiourea is excreted in the urine. As it has never been found in the urine beyond the third day, Danowski (5) concludes that it is bound in some tissue in concentration greater than is found throughout the rest of the body and may be released so slowly as not to be detected after the third day.

That thiourea is a highly reactive substance physiologically is shown by recent references in the literature in such widely separated fields as resistance of animals to low atmospheric pressures (10), as a protective agent for vitamin C (23), as a deleterious substance in the development of amphibians (9) and fish (8), as having insecticidal action on larvae of the housefly (17) and fungicidal action on orange decay (4), and finally as producing a stimulating effect on the germination of lettuce seeds at high temperature (24).

In investigations conducted at this Institute, we have been dealing with relatively small amounts of thiourea. The amount of thiourea taken in with the consumption of one pound of treated fruit tissue would not exceed 0.1 g. and would probably be nearer to 0.025 g. (6). The amounts required for treating fruit tissues are well below the minimal amount necessary to produce thyroid effects.

SUMMARY

Rats fed thiourea (NH_2CSNH_2, also called thiocarbamide) in addition to a complete diet throughout their adult life span for a period of more than three years in the drinking water at the rate of 1.72 mg., 6.88 mg., and 27.5 mg. per kg. of body weight daily showed normal growth, except

that they failed to gain in weight during the first ten days of the experiment in all concentrations. When the experiment was repeated it was found that rats fed the highest daily dose (27.5 mg. per kg.) in a complete food gained less per day than the checks over a period of 44 days. These results were in general agreement with the previous test.

The general appearance of the treated rats was normal when compared with the check.

Autopsies of rats revealed no pathologic change that could be attributed to thiourea, the highest dose fed being below the minimal dose usually required to cause marked thyroid enlargement.

Recently W. E. Griesbach, T. H. Kennedy, and H. D. Purves in an article entitled "Protective action of potassium iodide on thiourea poisoning in rats" (Nature **154**: 610–611. 1944.) report that a few individual rats are highly susceptible to thiourea and that those that survive the first treatment develop an immunity.

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